

Threat of calamity for world trade

It is properly ironic that the conference on European political union beginning this week should follow so quickly last week's collapse of the four-year effort by 107 governments to extend GATT.

WHY does simple computer equipment cost roughly three times as much in most European countries as in the United States? Why does a kilogram of rice cost more than twice as much in Japan as anywhere else? Why are boots and shoes more expensive in the United States than, say, in Portugal, or textiles more expensive than in Hong Kong? These are well publicized consequences of trade protection. European computer manufacturers are believed to deserve the protection afforded by a tariff, shoe and textile manufacturers in the United States are protected both by tariffs levied on imports and by quantitative restrictions on imports, similar to the quota restrictions by which the members of the European Communities (EC) keep Japanese motor-car imports at bay. Soon, there may be many more such examples.

The explanation is simply that the intergovernmental conference planned four years ago to cap efforts to extend the scope of the General Agreement on Tariffs and Trade (GATT) broke down last week in Brussels in a cloud of recrimination. The consequences could prove calamitous. Yet the hotel beds in Brussels will not have cooled off before they are occupied by European diplomats gathering this week for a conference on the political unity of Europe. (There is a parallel conference on monetary union.) The coincidence is apt because last week's damaging events are almost entirely attributable to the incapacity of the EC to act with political maturity. It is ironic because the collapse of the trade negotiations will itself diminish Europe's chances of winning the degree of political maturity that might have averted last week's catastrophe.

Catastrophe

The GATT affair is a catastrophe because it brings to a halt, at least for the time being, the process by which, in the past 40 years, the world has created previously unimagined wealth by practising internationally Adam Smith's eighteenth-century recipe for the wealth of a single nation, Britain as it happened. The precept is that of the division of labour, the concentration of particular tasks in the hands of those able to carry them out most effectively, and who are able and willing to do so most cheaply. In the long run, we shall all now be losers, but there will be more immediate disappointments for developing countries, for those such as pharmaceutical manufacturers who had been looking for more effective

worldwide protection of intellectual property and, for different reasons, the countries of Eastern Europe, still looking for the economic benefits of last year's freedom.

The much-advertised obstacle on which GATT stumbled last week was the EC's refusal to negotiate with the United States and other major food producers on its protection of European agriculture, estimated to cost Europeans \$46,000 million a year. But what the GATT negotiations most clearly demonstrated is that, when faced with the prospect that the negotiations would collapse, the European Commission (to which EC's trade negotiations are delegated) lacked the authority to cut a deal.

Subsidies

Constitutionally, the Commission is not a government, but, rather, a bunch of civil servants with responsibility for administering and enforcing the Treaty of Rome, of which the infamous Common Agricultural Policy is an integral part. Technically, EC decisions are made only by the Council of Ministers, a device by which all member governments are represented by ministers or even by heads of government (as at 'summit' meetings). That this system does not function well is plain from last week's collapse of the GATT negotiations. Decisions on European agricultural policy are made by agricultural ministers from member states, precisely those most acutely sensitive to the sectional demands of their own farmers. It was never realistic to expect such a group to improve on EC's first (and final) offer of a 15 per cent reduction of farming subsidies over the next five years added to the 15 per cent since 1986 forced on the EC by budget pressure. European heads of government could have broken the deadlock, but chose not to do so, which is entirely consistent with their refusal to accede to Mrs Margaret Thatcher's demand that they should discuss the then-impending collapse of GATT at their meeting a few weeks ago in Rome.

Yet, the ambitions of Europe's federalists notwithstanding, the intergovernmental conference on political unity begun this week (it may last for months) will almost certainly end up confirming the view put out at the weekend by the governments of France and Germany that the central role of the Council of Ministers should if anything be strengthened. The trouble is that ministerial councils are secret; those who attend them may simply



follow their national government's line without ever saying what it is. They may be accountable to their governments, but that does not count for much when there are good reasons to suspect that the member governments differ among themselves on important issues, the virtues of an extended GATT agreement included. Last week's collapse can only strengthen the fear that the EC's objective is to become a self-sufficient trading bloc isolated from the outside world by high tariffs and other antediluvian devices. And that can only strengthen the reluctance of free-trading governments, notably the British, to agree to even modest steps towards further union.

Fiasco

None of this implies that the Europeans were the sole cause of last week's fiasco. The United States is partly to blame. The idea that there should be a substantial reduction of agricultural subsidies goes back to a proposal of President Ronald Reagan in 1985 that they should be abolished altogether. It is an admirable objective, but one that could not be brought into being without enormous social and economic consequences. US insistence last week on a 75 per cent cut in the next five years was inflexible, exciting the canard that it had been designed to provoke rejection. Backtracking by the United States on its own proposal that the GATT agreement should be extended to trade in services similarly suggested that the United States regards GATT as an instrument of its domestic policy, an impression reinforced by the readiness of the United States to by-pass GATT, and to act unilaterally, on several recent occasions. But shamefully, Europe, and agriculture, are chiefly responsible for what happened last week.

What can be done to rescue something from the ashes of the past four years? Technically, if officials working at Geneva were able to cobble together an agreement by the end of February, there would still be time to push it through the US Congress before its own pre-set deadline expires. But if senior politicians failed to reach an acceptable compromise last week, what chance is there that international civil servants will be able to do the job instead? The best bet would be to tie up the loose ends in the uncontentious parts of last week's near-agreement, postponing agriculture, services and intellectual property for, say, a year. But for no longer. The devotion of the world's rich countries (Japan as well as the United States and those of Europe) to agricultural protection needlessly impoverishes their own people, but is also a cruel deprivation of developing countries, not to mention those such as Australia and New Zealand that have made the most persuasive case in the long GATT negotiations.

Agriculture is everybody's embarrassment. Less than 20 years after the UN World Food Conference at Rome sought to persuade the world that it was on the edge of famine, it emerges that there need never be a physical shortage of food. So why do people still go hungry? Because there are physical problems of distribution, as now (after a bumper harvest) in the Soviet Union. Because

productive agriculture, which has proved to be a capital-intensive business, is most of all productive in rich countries, not traditional rural economies. And because many poor countries cannot afford the prices that farmers in prosperous countries must charge for their produce to share the well-being of their countrymen. One remedy would be to help poor countries produce more and rich countries less. Sadly, decades of aid programmes have foundered on the first objective, while the second has been defeated by the assertion of farmers in rich countries that they and their offspring have an inalienable right to be farmers for the rest of time.

The US proposal in GATT would have moved the world in both directions, but too quickly for safety, let alone comfort — chiefly that of farmers in rich countries. But the complete abolition of farming subsidies in 20 years would be a realistic objective. Why not settle now on steady progress towards that end? Meanwhile, there is a strong case for taking the edge off the most contentious consequence of farmers' over-production — the practice of governments (and the European Commission) holding surplus stocks of food of dumping them on world markets at knockdown prices, sometimes simply to save the cost of storage, sometimes to win political favours. The United States has been complaining loudly for the past four years at the EC's export subsidies, which are explicit, but its own export prices have often been lower than farmers' production costs. This fairy-tale competition is not merely ruinous, but robs some poor countries of the incentive to grow food for themselves. Why not blunt the difficulty with a transitional agreement to sell surplus stocks in a seemly fashion, perhaps through an international marketing agency and an inverted auction?

There are also wider issues to be tackled. The failed deal in agriculture had been made a condition of agreement on intellectual property. Developing countries had declined to extend protection of rich-country patents and copyrights while their own agriculture is kept out of world trade. But GATT, whose guiding principle is that customers should not discriminate between potential suppliers — tariffs are not outlawed — is not the best forum for that wrangle. Reviving the negotiation on technology transfer in the UN Conference on Technology and Development, which has been stalled for the past eight years, would be a better route.

For members of the EC, there is the more urgent question of how Europe can be equipped politically to play a grown-up role in the international affairs in which it wishes to be engaged. Those discussing political union in the coming weeks will be eloquent about Europe's role on the world stage, but last week's failure will empty the rhetoric of meaning if the EC cannot equip itself to negotiate sensibly with its counterparts or to shake off the sectional interests behind the Common Agricultural Policy, which has long since outlived such utility it may have had in 1957. The fear that, in the present fragile economy of the world, the GATT failure may precipitate a slump should be a spur in that direction. □

Genome project to tackle mass screening

- Cystic fibrosis pilot planned
- Concern over genome map delays

Washington

PLANNERS on the Human Genome Project last week tentatively agreed to take the lead in a pilot project to screen the public for cystic fibrosis (CF) in what will be the first such project in the United States. At a meeting of the project's advisory committee, panel members advised project director James Watson to start looking for government and private partners in a pilot screening project.

Several of the National Institutes of Health (NIH) have expressed interest, as have other branches of the US government, including the Agency for Health Care Policy Research, a branch of the Public Health Service.

Scientists have been working to map and sequence the human DNA molecule for two years while widespread testing for genetic diseases — a major application of the research — has languished. But before widespread testing can begin, policy-makers must tackle legal and ethical issues that have prevented the setting up of any genetic screening trials in the United States while trials have gone ahead elsewhere.

Unlike countries such as the United Kingdom and Denmark which have already begun testing, the United States has no public health programme. The CF test is expensive — about \$300 per sample — beyond the pocket of many potential subjects.

With mass testing and greater coordination, those costs could drop. Fewer than 3,000 CF tests are at present processed each year in the United States. In contrast, Britain, where laboratories this year will process some 50,000 samples and where public health care pays for much of the education, counselling and follow-up service, the tests cost only \$2 each.

US policy-makers must also overcome a shortage of testing laboratories and genetic counsellors. A draft report from the genome project's working group on ethics, legal and social issues points out that "there are 3.5–4 million births in the United States each year. If even a small fraction of those requested CF testing, the system would be overwhelmed." There are fewer than 1,000 genetic counsellors in the United States, and only 75 new master's level counsellors are trained each year, the report notes.

These logistical problems are overshadowed by the abortion issue: critics claim that testing is simply a rationale for

abortion, leading some genetic disease organizations to shy away from screening as one controversy they can do without.

Although many private health insurers will now pay for genetic testing, some do so only with the agreement that the fetus will be aborted if it shows a high probability of developing a serious — and expensive — disease. Research into such discrimination against carriers of CF and other genetic diseases will be funded by the genome project — 4 per cent of the total budget is marked for ethics research.

The debate about CF is further complicated by the disease's complex genetic origin. The defective gene, discovered last year, is found in only some three-quarters of those who carry a genetic weakness for CF. Various mutations of the gene account for the rest. At best, current US tests can identify only 84 per cent of the carriers. Some groups have called for a moratorium on testing until a 95 per cent detection rate is reached, but without a research breakthrough, the 90 per cent rate now achieved by one Danish group may be the practical limit.

Deciding which of these issues are serious hurdles and which have simple solutions must wait for a large-scale pilot project. "These are all research questions and you need a research setting to answer them", says Nancy Wexler, who directs the genome project's ethics working group. "The main thing is that somebody says 'We have to take responsibility for these projects'". But some genome project planners are concerned that becoming involved in genetic screening could shift the emphasis of the effort from basic research to public policy — and delay the completion of the genome map.

Breast cancer and diabetes now seem to have genetic links, and a workable test would no doubt spur calls for widespread screening for them as well. "Once you let the camel's nose under the tent, how do you keep the whole camel out?" asked Norton Zinder, chairman of the genome advisory committee, last week.

Wexler, however, does not believe that pilot screening projects will divert the genome project from its goal. "Once you work out the questions for one disease, you're in a better position for the next one", she says. And funding for the CF pilot project, if approved, will come out of the 4 per cent ethics budget, not from research funds, she points out.

Christopher Anderson

US SPACE PROGRAMME

NASA woes are self-inflicted says report

Washington

THE future of the US space programme depends on cutting costs, setting new goals and scaling down big projects like Space Station Freedom, the Augustine commission on NASA's long term future reported on Monday.

Some concerns over NASA's activities are "deserved, and occasionally even self-inflicted", says the report.

The greatest flaw is that there is no clear purpose to the space programme. NASA has been trying to do too many different things with limited resources, and has contributed to its own downfall by underestimating project costs and safety margins, then cutting back smaller projects to keep the larger ambitions alive.

The committee says some goals must be set aside or postponed until money can be found to pay for them.

NASA's highest priority should be a science programme, using primarily unmanned launch vehicles. Next should be a Mission to Planet Earth, to provide comprehensive data for the global environmental debate, and only after that should manned space exploration be contemplated. The trip to Mars is a suitable long-term goal but it should be made of achievable steps which could be accomplished as money and technology allow.

In specific terms, the space shuttle should be retained as the basic means of ferrying people into space, but a new fleet of rockets should be the workhorses of the scientific space programme.

The space station will not immediately be needed as a transportation mode for manned exploration and should be rethought. Its chief purpose should be to conduct life science studies in weightless conditions, and the present 90-day redesign period ordered by Congress should be extended, if necessary, so that a space station with specific purposes and the ability to achieve them will be created.

The prospect of further redesigns does not trouble the Europeans at this stage, according to Ian Pryke of the European Space Agency's Washington office, who agrees that is more important to get things right than to stick to a rigid timetable.

The committee chaired by Norman Augustine, formerly head of the aerospace company Martin Marietta was set up six months ago at the request of US Vice-President Dan Quayle (see *Nature* 346, 206; 1990).

At that time the flaw in the Hubble Space Telescope had just come to light, the space shuttle fleet was grounded by persistent fuel leaks, and the feasibility of the space station was being questioned.

David Lindley

All to play for in Madrid

London

A controversial French and Australian proposal that would have made Antarctica a unique continental 'natural reserve' was rejected by delegates from the Antarctic Treaty nations, meeting in Vina del Mar, Chile, over the past three weeks. But the central plank of the French and Australian idea, a permanent ban on mineral exploitation in the continent, remains a live issue that is set to divide the 39 Antarctic nations once more, when they meet to continue negotiations in Madrid next April.

National delegates and environmentalist groups agree that the Chile meeting made some progress towards a framework for environmental protection in Antarctica. Environmentalists had dreaded a complete breakdown of negotiations, as the present voluntary ban on prospecting may be lifted if treaty members are no longer working towards an agreement on mining.

After more than two weeks of impasse, a draft protocol on environmental protection was put together by Rolf Andersen, head of the Norwegian delegation. Specific environmental issues, including mining, will be dealt with in separate annexes to the protocol. Scientists worried that new provisions to assess the environmental impact of research will restrict their work (see *Nature* 348, 269; 22 November 1990), must now wait until the Madrid meeting, or later, before that annex is finalized.

Paul Bogart, from Greenpeace International, says that negotiations progressed "at a snail's pace, but at least the snail is moving in the right direction". Environmentalists are pleased that the Conven-

tion on the Regulation of Antarctic Mineral Resource Activities (CRAMRA), which would have allowed mineral exploitation in the future if all treaty members agreed to it, has been superseded by the plan to address the issue in the new protocol.

But other experts say the demise of CRAMRA may be bad for Antarctic environmental protection in the long run. Treaty members may agree a moratorium on mineral exploitation for 30 years or more as a compromise solution (practical difficulties in any case will preclude mining or oil exploration in the near future). But US officials say that some ground rules to control exploitation must be drawn up to prevent the free-for-all that might ensue when any moratorium expires.

Ron Naveen, from Oceanites, a US educational foundation which concentrates on marine and island environmental issues, believes that some of the "positive aspects" of CRAMRA should be used as the basis for a minerals annex to the new protocol. The need for consensus agreement before allowing mineral exploitation could be extended to prospecting, he says, to strengthen environmental protection.

This will require a more flexible approach than the entrenched positions that dominated in Chile. The rigid French and Australian support for a total mining ban was opposed implacably by Japan, which is poor in minerals reserves and is CRAMRA's strongest supporter. British officials, while less inflexible than the Japanese, still have strong reservations about a permanent mining ban.

Peter Aldhous

ENVIRONMENTAL RESEARCH

Global research centres open

London

Imperial College, London, and the University of Oxford are both launching new environmental research centres in the hope that the recent high profile given to environmental issues will win funding from industry.

Imperial's Global Environment Research Centre opens tomorrow (14 December), with six staff and about £1 million of start-up funds from the college. But director Iain Thornton wants to recruit ten postdoctoral researchers and set up Britain's largest environmental library, which will require £12 million over the next five years. Thornton is seeking donations from companies in Britain, North America and Japan.

The Imperial centre will carry out research into climate change, pollution and

clean technology, and will also develop environmental policy. Director of policy John Gordon, a former diplomat, hopes to use seminars to bring together policymakers, industrialists and pressure groups. An independent voice is needed to ease the often confrontational relationship between government and environmentalist groups, he says.

Oxford's Environmental Change Unit will open in February, concentrating on the impact of environmental change on forestry and natural ecosystems. The university has again provided initial funding of more than £1 million, and the US computer company IBM has agreed to pay the director's salary and provide computing facilities. But another £5 million is still needed from industry.

Peter Aldhous

France gets greener

Paris

THE French National Assembly has approved a series of measures that will lead to the formation of a national environment institute and of a trio of government agencies with a brief to cut pollution and save energy.

The new environment institute, to be located at Cergy-Pontoise near Paris, will unite knowhow and data on environmental issues. According to the French environment minister Brice Lalonde, France has until now had a "do-it-yourself" system of expertise operating in each discipline. This has obscured a clear public image of environmental matters, and has been the source of "considerable distortion and financial waste".

France has only recently turned its mind to green matters, but is now acting with gusto. In the last budget, Lalonde was given a separate ministry, with a 40 per cent increase in funding. In June this year, he announced a more "aggressive" environment policy.

The French arm of the environmental group Greenpeace welcomes the initiatives, but is sceptical of their real impact. "It cannot do any harm", said a spokeswoman, "but it is more of a political facade designed to clear consciences."

Peter Coles

STANFORD UNIVERSITY

Tacking on grants

San Francisco

IN the latest episode of Stanford University's ongoing saga of problems with the high overhead rate it charges to government research grants, Stanford has admitted that in the name of research it billed taxpayers from 1981 to 1988 for depreciation of its 72-foot yacht, the *Victoria*, as well as for smaller sailboats, sculls and other equipment. The charges of \$184,286, which University officials attribute to an accounting error, have been repaid by reducing Stanford's claim for overhead expenses owed by the government, the University announced last week.

The charges came to light after queries by a US Congressional committee which is investigating university overhead rates, focusing on Stanford's 74 per cent addition to every research grant application. In October Stanford denied that any expenses related to the vessel were billed to the government. After additional probing by the General Accounting Office, however, the University said it had been mistaken and the yacht's depreciation, though not its operating costs, had been charged.

As Congressional inquiries continue, the *Victoria*, donated to Stanford in 1988, remains anchored in the San Francisco Bay, where it is being offered for sale for \$475,000.

Elizabeth Schaefer

ASTRONOMY

Per ardua ad astra

Washington

DESPITE a troublesome start, the orbiting astronomy package Astro-1, a set of telescopes tucked into the cargo bay of space shuttle Columbia, was pronounced a great success halfway through its ten-day mission.

The threat of a premature end to the mission, after the second of two instrument display units in the shuttle cabin failed, was averted when control of the three ultraviolet and one X-ray telescopes was transferred to the Marshall Space Flight Center (MSFC) in Huntsville, Alabama. A makeshift arrangement, with astronauts steering the telescopes manually and scientists at Marshall operating them, was working smoothly in less than a day. Last weekend, there was some hope that the lost day could be recovered by the extension of the mission to an eleventh day but over the weekend a waste liquid tube became clogged, and it seemed as if the shuttle would have to return to Earth early. By last Monday the plumbing was fixed, but by then the threat of bad weather in California and Florida forced a decision to land early anyway.

The Astro-1 observatory was due to have been launched in May, but was delayed by the plague of hydrogen leaks that afflicted the shuttle fleet throughout the summer. Then, after the successful launch of Columbia on 25 November, a string of problems hinted that Astro-1's ill-fortune might continue.

One of two units displaying the status of the astronomy package failed altogether after nine hours, apparently due to overheating. A partial computer failure in the Wisconsin Ultraviolet Photo-Polarimeter Experiment (WUPPE) set back attempts jointly to align all three ultraviolet telescopes, and a later computer crash disabled an optical tracking device needed to lock on to guide stars and maintain the telescopes' pointing accuracy.

But all these setbacks proved temporary or manageable. By the third day of the mission, all four telescopes, including the separately steerable Broad-Band X-ray Telescope (BBXRT), were working satisfactorily and, with manual help from a mission specialist operating an on-board joystick, were being pointed successfully and held on target.

But after a full day of observation, the second of the two instrument display units failed, apparently also from overheating. This left the astronauts able to steer the telescopes, but unable to perform the necessary switching of filters and management of exposure time.

Throughout last Thursday, mission scientists worked to re-route control of telescope operations to MSFC, and were able to try out a first version of the new operating regime barely 12 hours after the

failure. By Thursday evening, Astro-1 was up and running again. At the same time, the observing schedule was reorganized: each 12-hour block of observing time was planned just 12 hours in advance.

With control of the observatory now in the hands of principal investigators at MSFC rather than the mission specialists on Columbia, some astronomers say that the new mode of operation is preferable to that originally planned.

Bill O'Connell, of the Ultraviolet Imaging Telescope (UIT) team, says that, in general, fewer objects are now being observed for longer, which in fact meant more science for UIT, a camera whose film will not be developed until after Columbia returns. In the original plan, there were many short observations which enabled WUPPE and HUT to take quick looks at a variety of interesting objects, but which were too short to allow UIT to obtain useful images.

According to Art Davidsen, of the HUT team, all the highest priority objects for their instrument would be successfully rescheduled. Halfway through the mis-

sion, HUT had already obtained "lots of good stuff" on quasars and galaxies. One of the most exciting possibilities for Astro-1's ultraviolet observations, which reach to short wavelengths never before observed, would be to gather evidence for emission from extremely hot gas very close to the giant black holes thought to power quasars and active galaxies.

Steve Maran, of the UIT team, called Astro-1 an "extraordinary success", and said that operations were becoming more efficient with every day. The fact that the mission had recovered so rapidly and completely from a variety of difficulties argued in favour of this kind of science effort on the shuttle, in which observers operating their own instruments could adapt quickly to changing circumstances.

Whether there will indeed be more has yet to be decided by the National Aeronautics and Space Administration (NASA). A new shuttle manifest, covering the next three years, was released last week, with no spot for a reflight of Astro-1. But Davidsen is hopeful that astronomers will push hard to make it fly again, and he hopes that even NASA "will budge when they see the results."

David Lindley

OPTICAL ASTRONOMY

Telescope high and dry in the desert

Munich

ONE of the clearest, driest places in the world was chosen last week by the European Southern Observatory (ESO) as the site for its Very Large Telescope (VLT). Cerro Paranal, a 2,664-metre-high mountain in the Atacama Desert in Chile, was

ESO

IMAGE UNAVAILABLE FOR COPYRIGHT REASONS

The best site for sight selected for the excellent image quality and good weather as well as the absence of light pollution.

A six-year meteorological survey showed that Paranal is one of the best sites in the world for optical astronomy, approaching the standards of Mauna Kea, Hawaii, generally considered the best year-round site for optical and infrared astronomy. Several US and international telescopes are based there.

Although Mauna Kea has the advantage of being at a higher altitude, so that less

atmospheric water vapour interferes with spectroscopic measurements in the infrared, Paranal has more clear nights and a more stable atmosphere. In terms of 'seeing', meaning the sharpness of stellar objects in both optical and infrared astronomy, Paranal will be better (at a median of 0.66 arcseconds) than the main ESO facility in Chile at La Silla (median of 0.76 arcseconds), which is well known for its excellent observing conditions. Similar measurements are not yet available for seeing at Mauna Kea.

The only surprise about the decision was the placement of the telescope so far from La Silla, 600 km to the south. ESO astronomer Massimo Tarenghi was confident that no logistical difficulties would arise from the separation.

VLT consists of four 8.2-metre telescopes which can work separately or together, in which case they make VLT with its effective 16-metre diameter the largest optical telescope in the world. The four telescopes will begin to be installed in 1995 and the entire array should be in place by late 1998.

There will be a satellite link between Paranal and ESO headquarters in Garching, Germany, allowing astronomers to control VLT remotely. The high-speed link of up to 8 megabaud will allow even better remote control than is currently possible between Garching and La Silla.

Steven Dickman

Japan takes US role model

Tokyo

AN influential society of Japanese academics and industrialists recommended on Monday that the Japanese government should form an equivalent of the US Office of Technology Assessment (OTA) to oversee science and technology policy and in particular large-scale international research projects, such as Japan's participation in the US space station and global environment research. But the proposal is bound to meet strong resistance in certain government circles.

The Japan Society of Technology, which made the proposal in a report entitled *A proposal concerning technology and human welfare: toward building a harmonious global society*, consists of nearly 70 senior academics and industrialists, including Haruo Yamaguchi, chairman of Nippon Telegraph and Telephone Corporation, Michio Okamoto, former member of the Council of Science and Technology, Japan's leading science policy-making body which is chaired by the prime minister, and Jiro Kondo, president of the Science Council of Japan (JSC), the leading academic advisory council of the government. An *ad hoc* committee of the society took two years and about 20 meetings to compile the report, which has been submitted to Prime Minister Toshiki Kaifu and to heads of all science-related ministries and agencies.

Kondo, who chaired the *ad hoc* committee, points to the US request for Japan to participate in the Superconducting Super Collider (SSC) project as an example of the sort of global research issues the proposed Japanese OTA would tackle. Since the US Department of Energy officially invited Japan to join the SSC project in June, the Japanese government has been paralysed by the inability of any of the government ministries and agencies involved to take a lead and reply to the US request.

What is needed, says Kondo, is a public organization that includes scientists and specialists in the humanities and social sciences to assess the social as well as the scientific and technological value of such projects. But the government has so far made no attempt to ask academics what they think of the SSC, he says. In contrast, he cites Japan's Human Frontier Science Program as an example of a successful international project that included significant input from Japanese academics.

Kondo says the proposed technology assessment office would also look at domestic government research projects and where necessary recommend termination. He gives the nuclear-powered ship *Mutsu* as an example — after more than 20 years and an expenditure of about \$1,000 million, *Mutsu* has spent only a few days at

sea and will be scrapped next year.

But the proposal is intended to be much more than just a recommendation to form a domestic Japanese OTA. The society's ultimate aim is the establishment of an international body like the United Nations that would oversee global technology projects and Overseas Development Aid in an attempt to ensure that technology benefits all mankind. But before that lofty goal can be achieved, Japan will have to put its own house in order.

Although many people in Japanese government and academic institutions have long agreed that there is an urgent need for an independent watchdog to monitor government science and technology policy, all attempts to form such an organization have failed. The JSC, when established 40 years ago, was supposed to fill such a role. But after about 20 years, the ruling conservative Liberal Democratic Party (LDP) became convinced that the council was run by "a nest of communists",

says Kondo, because it often disagreed with government policy. And in 1985 the system of election of council members was restructured to allow the prime minister to screen all candidates.

The Science and Technology Council chaired by the prime minister is now the principal organization for setting policy. But the council is closely related to the Science and Technology Agency, in which its office is located, and as a result its independence is open to question.

Kondo believes that JSC could organize the proposed assessment office if it were given a budget. But the JSC budget has been frozen since 1985 and although relations have improved with the LDP, there are still some members of the ruling party who are suspicious of the council's links with communist countries.

Tomorrow (14 December), an advisory council of the Science and Technology Council is expected to publish a separate series of recommendations for dealing with global science and technology issues. And with two reports in one week, debate of this controversial issue is bound to heat up.

David Swinbanks

Public funds for POST?

London & Munich

THE UK Parliamentary Office of Science and Technology (POST), set up in April 1989 to inform Members of Parliament (MPs) on scientific issues, may win support from public funds following the change in prime minister.

After former prime minister Margaret Thatcher refused to provide any public money, POST's founder, the Conservative MP Sir Ian Lloyd, turned to companies and charitable foundations for donations. A number of MPs (ironically, including Thatcher herself) have also provided smaller sums from their own pockets. But Lloyd says he was able to press POST's claim for public funding with the three

candidates to succeed Thatcher, as they vied for the support of Conservative MPs in the party's leadership election. The new prime minister John Major is said to have an open mind on POST's funding.

POST expects to spend about £150,000 in the coming year, producing ten briefing notes for MPs. Three reports, in the style of those from the US Office of Technology Assessment (OTA), on computing in schools, research in the National Health Service and the balance between civil and defence research spending, are also being prepared. Lloyd says that POST is "quite unashamedly modelled on the OTA", but with a permanent staff of only four, POST's activities are severely limited. Unlike POST, however, both OTA and the science policy division of the Congressional Research Service (which provides a similar service to POST's briefing notes) are publicly funded.

Other European countries fund similar, if smaller, organizations, says Michael Norton, POST's director. The German parliament set up a Technology Assessment Office last June, giving it a budget of over DM 2.5 million (about £870,000) a year, for a three-year trial period.

Lloyd says the first step is to get public funds for POST to match private donations. Changes to the mechanism of parliament happen very slowly, but he hopes for a "positive development" before the next general election.

Peter Aldhous & Steven Dickman

ACADEMIC FREEDOM

Clampdown continues

London

MONEIM Attia, the Sudanese environmental physiologist imprisoned without trial since January (see *Nature* 348, 5; 1 November 1990), has now been released. But Alex de Waal, from the human rights pressure group Africa Watch, says that the abuse of academic freedom by the governing Sudanese revolutionary council continues unabated. Most political detainees are released after several weeks or months, he says, and a number have since been re-arrested. About 130 university lecturers have been dismissed in the past three weeks, apparently on political or ideological grounds.

Peter Aldhous

Luring back the exiles

Budapest

THE Hungarian research community is anxiously awaiting a decision later this month that could signal the beginning of a scientific renaissance. But its hopes are clouded by economic and other uncertainties.

At issue is a government proposal to double the Hungarian Research Fund (HRF) from 3,800 to 8,000 million forint (\$140 million) over the next four years. If the proposal is approved, it will be a first step towards improving the country's economy through research, and, it is hoped, encouraging many of the large number of scientist emigrés to return to Hungary.

Like other new democracies in Eastern Europe, Hungary is entering a period of difficult choices, during which the inevitable economic and environmental crises will make it difficult to focus on improving the country's scientific base.

In some ways, Hungary is worse off than its neighbours. It is saddled with a foreign debt of \$18,000 million, more than any country in the region except Poland, while its 15-year start on a limited form of capitalism has exhausted many researchers, who have found it necessary hold second jobs.

But Hungary also has several advantages, including a long tradition of scientific excellence and a young population that is positively passionate about science. The past 15 years of relative openness have also brought rewards, such as better contacts for those who have stayed at home and a rich pool of successful Hungarians abroad.

The World Bank recognized this potential when it proposed a 'Human Resources Project', supported by a loan of at least \$100 million and likely to be approved in March 1991. Much of the money will be used to train young scientists, in contrast with projects in Poland and Romania which are focused on unemployment and health issues.

Within the government, there is strong political support for science. "The government understands that science and technology are the keys to a breakthrough for our economy", declares Ferenc Madl, minister without portfolio responsible for science. "We are committed to science and we have access to skilled people", he adds. "The basic problem is money."

Money is everyone's problem in Hungary. Uncertainty hangs over the research community, like society in general, because of inflation, which is officially acknowledged to be 30 per cent a year, but which some estimate to be 50 per cent. Economic difficulties have been further increased by a drop in exports and the loss of cheap oil from the Soviet Union. Support for October's blockade of

all roads into and out of Hungary by taxi drivers protesting at higher petrol prices is seen as a signal that the government must aim at as smooth a transition as possible to a market economy.

In this light, the proposed doubling of the HRF seems remarkably generous, as long as the amounts are adjusted each year for inflation.

Madl has also persuaded the government to allow HRF funds to be drawn directly from the proceeds of a special 4.5 per cent tax on the profits of private businesses. Previously, the tax receipts of 8,000 million forint a year were used only for a fund for new technology. This change must also be approved by the parliament.

Unfortunately, the picture is not everywhere as rosy. The proposed salary increase for employees of the Academy of Sciences and universities is just 20 per cent and the budget for operating costs will rise only 10 per cent, far short of the "dream" figures of 30 and 50 per cent, respectively,



The Hungarian Academy of Sciences, Budapest, where "little castles" are in need of toppling, according to Ferenc Madl (right).

proposed by Istvan Lang, secretary-general of the academy. At their current salaries, Lang explains, many researchers have to moonlight to make ends meet. "We will fight for more", he says.

Despite the promised increases, many researchers view the changes with suspicion. One young researcher, in his former institute on a brief visit from his current foreign post, summed up the general scepticism towards the promised increases: science, he says, "will be the first to be cut" if the budget shrinks.

And money is not the only problem. Researchers fear that the dominance of the academy over the universities will be reinforced if the government does not soon initiate a thorough reform. Madl agrees, saying he would like to "institutionalize" cooperation between the academy and the universities so that the former does more teaching and the universities gain freer access to research equipment.

Madl admits that many institute directors, especially in the academy, are

jealously unwilling to "give up their little castles". But he forswears radical changes. The separation between the academy and the universities can be bridged only gradually, he says, on a case-by-case basis.

The academy answered some of these concerns at a meeting earlier this month, when it decided to select institute directors using more democratic processes, following up on efforts begun before the revolution.

Related to concerns about the power of the academy are suspicions that a group of insiders, not necessarily Communist Party members but rather an 'old-boy network', plays an inordinately large role in dividing up both domestic and foreign money.

For example, there are criticisms of the



HRF, which will become independent of the academy on 1 January, for allegedly unfair granting procedures that are said to favour politically powerful researchers at the expense of younger and potentially better-qualified people.

"Without an older sponsor", says one biologist, "it is impossible to receive money."

He says that some members of the grant-making committees read only the names, not the proposals, in deciding who should be given money.

There is also confusion about a rumoured grant of 30 million ECU from the European Communities for materials research which, it is said, is being distributed by only five people without procedures for outside review.

Madl says he "cannot imagine that this is true", but he accepts that the system might not yet be working perfectly. "Making peer review work properly is a problem even in the West", he says.

Nadl pleads for patience from the scientific refugees, acknowledging that only a prosperous economy will tempt them back. But time is running short. "A generation of our best students is now in graduate school abroad", says Alexander Szalay, an astrophysics professor at Eötvös University. "They will decide in three or four years if they want to return. If we lose them, it will take us 15 years to replace them."

Steven Dickman

BST gets clean bill of health

Washington

MILK and meat derived from cows treated with a synthetic version of bovine somatotropin, otherwise known as growth hormone, are safe for human consumption.

But despite the approval from experts at a National Institutes of Health (NIH) technology assessment conference last week, the more than five years of controversy surrounding bovine somatotropin is unlikely to end here. The drug has yet to be approved for commercial use by the US Food and Drug Administration (FDA) and the outcome of several investigations into scientific misconduct involving FDA and the drug companies are still pending.

Turning up the heat on the issue last week, the Consumer Policy Institute (CPI) released a report entitled *Biotechnology and Milk: Benefit or Threat* that called on the FDA to halt the sale of milk

from cows being experimentally treated with rbST, which FDA approved in 1985, while re-evaluating the impact of rbST on human health. CPI's report was met with an immediate rebuttal by the American Farm Bureau Federation (AFBF), the largest farm organization in the United States.

Despite the reluctance of NIH to become involved in regulatory matters, NIH convened the *ad hoc* 13-member panel of medical and veterinary experts at the behest of Congress.

The NIH panel concluded that the overall nutritional composition and quality of milk and meat from rbST-treated cows is equal to that of untreated cows and that the health of dairy cows is not appreciably affected. Acknowledging that some animal trials showed evidence of an increased incidence of mastitis, the panel

could not rule out the possibility that this might lead to an increased use of antibiotics and, thus increased antibiotic residues in milk.

During the lively scientific debate, panel members listened to disquieting claims, made by several speakers, of intimidation by drug companies and attempts by them and the FDA to suppress and manipulate animal health data on rbST. David Kronfeld, veterinarian at the Virginia Polytechnic Institute, said that he tried unsuccessfully to obtain sponsorship from two drug companies to study possible stress parameters in rbST-treated cows. University scientists are chosen Kornfeld said, "if they belong to the persuasion that rbST can have no adverse effects". Kornfeld also spoke of letters recently sent to the dean of his institute from a company scientist, which stated that he [Kornfeld] "should not be allowed to speak on bST, or that company would not give money to Virginia Tech".

Samuel Epstein, outspoken critic of rbST and professor of occupational and environmental medicine at the University of Illinois, called the panel's findings a "whitewash". Epstein alleges that confidential drug company research data in his possession clearly demonstrate that cows treated with rbST experience reproductive and general health problems, details of which, Epstein claims, do not appear in the published scientific literature. These allegations have prompted the House Committee on Government Operations to initiate an investigation of FDA by the Inspector General of the Department of Health and Human Services.

Diane Gershon

COMPUTER SECURITY

Networks spur to crime

Washington

THE dizzying growth of computer networks in the past decade has meant gaping security holes, primitive software protection and general disregard of the threat of computer crime, warns a report from the US National Academy of Sciences (NAS), published last week*.

Rather than being exceptional, the well-publicized cases of computer virus, break-ins and fraud that have captured headlines over the past few years are just the exposed tip of a growing computer security problem.

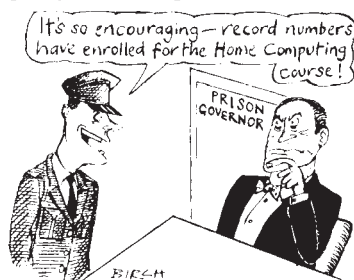
"So far, the nation has been remarkably lucky in escaping any successful systematic attempts to subvert critical computing systems", says David Clark of the Massachusetts Institute of Technology who headed the independent NAS panel. "Unfortunately, there is reason to believe that our luck may soon run out unless we take action now."

Systematic or not, the examples cited by the report are sobering: A \$259-million fraud in which staff at the car company Volkswagen reprogrammed computers to show false currency exchange transactions; an "almost-successful" attempt to defraud the Pennsylvania lottery out of \$15.2 million by breaking into a database of unclaimed winning tickets; and a foiled attempt to make and use thousands of automatic teller machine cards with personal identification numbers pirated from an online database.

The NAS panel calls for the creation of a non-profit consortium of computer users, manufacturers and others in the computer industry to research computer

security, track computer crime and promote adherence to an industry-wide set of security standards. The Information Security Foundation could operate on a \$15-\$20 million annual budget with funding provided by its members, NAS suggests.

Apathy and misguided trust are the



most serious obstacles to improved security practices, the report says. "It is as if a small town were to wake up one day to find that it had turned into a metropolis, yet everyone continued to leave their houses and cars unlocked based on blind faith in a past reality", says Clark. Much of the computer security now practised is carried out only so as to meet government regulations, he noted. And although some computer manufacturers are planning to adopt new protection techniques, others are reluctant to include security features — at extra cost and trouble — without greater customer demand.

But if US industry does not adapt common security standards soon, it may find it increasingly difficult to compete in the European market, where the European Communities are likely to set continent-wide guidelines for data protection, the report warns.

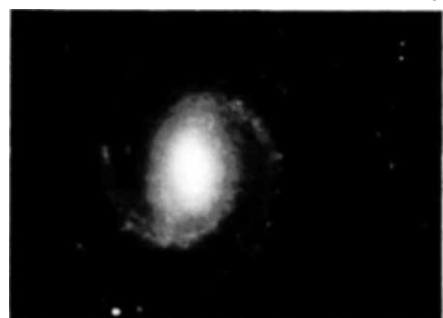
Christopher Anderson

KECK TELESCOPE

Mirror design success

San Francisco

THE innovative segmented-mirror design of the partially completed Keck Telescope has handily passed its first crucial test, say



its developers at the California Association for Research in Astronomy (CARA). The group hailed this "first-light" photograph of spiral galaxy NGC 1232 produced by the telescope on 24 November as evidence of the viability of the expensive and previously untried technology.

Elizabeth Schaefer

* *Computers at Risk*, National Academy Press, 1991.

FORENSIC SCIENCE

Horse dope-dispute rages on

London

THE horse-doping dispute between the Aga Khan and the Jockey Club, which regulates horseracing in Britain, has thrown a pall of gloom over the British horseracing community, chiefly because of the injured owner's decision to move his collection of 90 racehorses elsewhere. But the dispute may be the best thing yet to have happened to equine metabolic studies.

The dispute centres on the Aga Khan's horse Aliya, which came first in a race called the Oaks at Epsom, southwest of London, on 10 June 1989. Random testing at the Horseracing Forensic Laboratory (HFL) at Newmarket found in the horse's urine detectable amounts of 3-hydroxycamphor, a metabolite of camphor which is itself believed to be a stimulant of the central nervous and respiratory systems. Aliya was disqualified by the Jockey Club at the end of last month.

In the intervening eighteen months, both the reliability of the Jockey Club's screening methods and its interpretation of its data have been seriously criticized, mostly on the basis of investigations at the University of California at Davis and at the University of Quebec at Montreal, all commissioned by the Aga Khan.

The original complaint against Aliya, lodged 10 days after the race, was based

on a gas-chromatography mass-spectrometry assay used at HFL for screening urine and blood samples. It seems not to be disputed that detectable quantities of 3-hydroxycamphor were found in the urine, but no quantitative estimate was made. The detection limit is believed to have been about 125 nanograms per millilitre.

Professor Robert Massé, deputy director of the Canadian Center for Doping Control at Montreal (which is mostly concerned with human athletes) says that screening assays alone are insufficient to support a positive dope finding, and that they should always be followed by assays designed for the suspect substances.

Massé also says that the analyses at HFL were not as reproducible as they should have been. Mr Neville Dunnett, director of the laboratory, explains that by the need for haste in meeting a deadline for the adjourned Jockey Club enquiry.

But the more interesting disagreement centres on the origin of 3-hydroxycamphor in horses' urine. Gary Henderson from Davis says that feeding trials with horses there have shown that the metabolite can arise from naturally occurring substances other than camphor, including the closely related monoterpenes borneol and the wood constituents α and β pinene. Massé says

that horses bedded on wood shavings at the stables from which Aliya raced have been found to have the metabolite in their urine.

Part of the panel's case against the Jockey Club's decision is that, in circumstances in which the metabolite can arise from materials in the horses' environment, there should at least be a threshold concentration to determine when it has arisen unnaturally. Such a limit now exists for the finding of salicylic acid in a horse's urine.

Dunnett says that that the Jockey Club's rules are deliberately "black and white" to protect the "integrity of British racing". The rule is that a horse with a prohibited substance in its blood should not be racing even if the material got there by some natural route. "Occasionally, it may be a harsh rule", he says.

John Maddox

INDUSTRIAL/ACADEMIC COLLABORATION —

Rule change revives LINK

London

THE UK Department of Trade and Industry (DTI) will no longer discourage applications to the government's LINK research programme involving only one company. The change, announced by industry minister Lord Hesketh, is designed to increase participation by small companies, which have been discouraged by the perceived domination of projects by large companies.

LINK was set up in 1988 to bring academic and industrial researchers together in collaborative projects, but has been slow to get off the ground. Less than £40 million of the £210 million the government had hoped to spend by 1992-93 has so far been earmarked for specific projects, and the strict rules that govern the programme have been blamed.

Other changes are expected to follow. A team of independent consultants is now looking for ways to increase the uptake of LINK money, and expects to report to LINK's steering committee before Christmas.

The programme's critics argue that the selection of LINK projects could be streamlined, and that the government should contribute more than the 50 per cent it currently gives towards the cost of each project. Where a large proportion of a LINK project is university based, the 50 per cent limit means that industrial partners have to bear most of their own costs. Other similar initiatives, such as the European Communities' ESPRIT information technology programme, provide a powerful incentive for companies to take part by paying half of their research costs.

Peter Aldhous

SOVIET SPACE PROGRAMME

Commercial sponsorship reaches new heights

Tokyo

JAPANESE journalist Toyohiro Akiyama returned safely to Earth on Monday after propelling the Soviet space industry into a new, commercial orbit.

Lenin must have turned in his grave when a Soyuz rocket, the pride of the Communist nation's space fleet, carrying Akiyama of Tokyo Broadcasting System (TBS) network, headed for the sky adorned with the logos of numerous Japanese sponsors, including a leading manufacturer of sanitary napkins and paper diapers.

Japanese companies are said to have paid about \$30 million to sponsor the flight of the first Japanese into space, among them Sony, Minolta, Otsuka Pharmaceutical and Unicharm, the diaper maker. And TBS itself has spent \$37 million on the event celebrating its fortieth anniversary, including a \$12-million return ticket for Akiyama.

More than 120 TBS staff and engineers covered on the once top-secret Baikonur Cosmodrome in the Soviet Union to cover the launch and Akiyama's journey into space. And the journalist's daily descriptions on radio and television of space food, space sickness and the problems of

urinating and defecating in the MIR space station have really brought space exploration down to Earth and into the living rooms of ordinary Japanese.



Not satisfied with the \$12 million fee for Akiyama's space trip, Energia, the Soviet company that builds the Soyuz rockets and negotiated the contract with TBS, is said to have asked for an extra \$100,000 an hour for the services of Soviet cosmonauts who nurse-maided Akiyama though his flight. But TBS is resisting. The prime-time coverage of the event on TBS will undoubtedly help the Soviet Union's attempts to commercialize and cash in on its space industry. David Swinbanks

Catching the tide of innovation

Alfred Scheidegger

The reasons for Japanese pre-eminence in many areas of technology are widely misunderstood in Western countries, who would do well to take advantage of the opportunities that Japan offers.

JAPAN already dominates several fields of technology and is preparing to conquer others. The response to this challenge by Western countries, in particular the United States, is usually to complain that Japan is taking a free ride on Western science and technology. But researchers and especially industrialists would do well to stop grumbling and start taking advantage of the many opportunities now existing to tap Japanese expertise.

Participation

Earlier this year, the Japanese Ministry of International Trade and Industry (MITI), which has done much to foster the upsurge of Japanese industry by coordinating and supporting numerous research and development consortia, announced that foreign companies were authorized to participate in its major cooperative projects. In September, MITI offered foreign companies the opportunity to play a part in three large projects in which it plans to invest several hundred million dollars over the next nine years. Yet few, if any, businesses from outside Japan are expected to take up the invitation.

Not all the opportunities offered by Japan are attractive to foreign companies. The national genetic engineering project started by MITI in 1981 was certainly successful in developing this technology in Japanese industry and universities, where the basic know-how was almost totally lacking; but it would probably have been less useful to foreign participants. Other projects, however, such as that to develop environmentally compatible manufacturing technologies, would be highly rewarding. Interdisciplinary research consortia have been created to recruit the various specialists necessary for the development of new, complex technologies, which would otherwise be difficult to accomplish. The amalgamation of diverse technologies in this way is one of the strengths of the Japanese approach, and has led to their discovery and leadership of fields such as opto-electronics and mechatronics. In addition to the national projects, many of the universities are offering to supply research findings whose commercial value is not always immediately obvious, or for whose exploitation no suitable Japanese partners can easily be found.

Any foreign company wishing to avail itself of Japanese expertise in science and technology must thoroughly understand

both the nation's research system and its people's approach to life and work. Applied, practically orientated and technical research is much more in tune with the Japanese disposition than is basic research, even though their basic research seems to be advancing. Indeed, Japanese papers are now among the most often cited in physics and biology. But the percentage of engineers graduating from institutes of higher education in Japan is five times higher than in any other country in the world, indicating the enduring Japanese partiality for the technical and the practical.

That Japan's research and development expenses are rising three times faster than those of the United States should help to dispel any lingering doubt that Japan is determined to take the lead in science and technology. But money alone is not the key to rapid advances in technology. Japan's financial commitment is backed by a unique interaction of governmental and private leadership, allowing competing firms to collaborate successfully in research projects.

Biotechnology is a particular instance. For companies that are hampered by political constraints in their home countries, Japan offers an unusually favourable environment. Unlike some European countries, it has no laws restricting the application of gene technology, although five different ministerial guidelines have been issued on the use of recombinant DNA. With the exception of the Environment Agency, all ministries concerned regard the present guidelines as adequate, because no accidents have occurred since modern industrial biotechnology was introduced to Japan ten years ago. Political opposition to biotechnology is virtually absent, as attested to by the recent unhindered establishment of many biotechnological research facilities, and of production plants for drugs manufactured with the help of genetically engineered organisms, for example Toyobo's new pilot and production plants in the Shiga Prefecture. In harmony with OECD guidelines, MITI expects organisms that are non-pathogenic in industrial processes to be treated in accordance with ministerial guidelines on good, large-scale industrial practice. But the Japanese definition of a genetically changed organism differs from those adopted in other countries, relating only to the introduction of foreign DNA, and not to chromo-

somal or cellular manipulations.

Some large foreign companies are setting up or already operating research laboratories in Japan in the hope that their new discoveries will be imbued with the Japanese flair for application. But few have so far gained unqualified acceptance as part of the research community. It is hard for job-rotating foreign nationals in key positions to acquire appreciation of the very different Japanese code of business ethics. Nevertheless, with that appreciation it is surprisingly easy to collaborate with one of the university laboratories: for example CIBA-GEIGY's newly established International Research Laboratories in Japan are engaged in several cooperative projects with universities, and not only has the transfer of technological expertise been quick and easy, but long-term collaborative enterprises are simple to begin because of the existence of special centres for cooperation at universities (for example the Centre for Development of Cooperative Research at Kobe University, or the Seiken Institute at Kyoto University).

Commitment

Foreign companies wishing to join with Japan in a national research consortium have to make a great deal of effort and a strong commitment. Any attempt to evade or curtail the all-important preparatory phase involving lengthy negotiations and many workshops is taken by the Japanese as an affront; and companies looking for quick commercial successes in return for their participation will have come to the wrong address. The research consortia are concerned with high-risk, next-generation technologies, and profits cannot be expected for at least 10 to 20 years. It is this forethought that has led to MITI initiating neuro- and optical-computing projects due to start in 1992. Western scientists often look condescendingly upon such undertakings, which sometimes even lack a clear strategy; but who else is developing parallel-computing neural networks with an investment of several million dollars, so as to be able to compete with the Japanese in 20 years' time? Ignoring Japan's opening doors may sooner or later turn out to be committing technological suicide. □

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Roots of dogma in biology

SIR—I respond to the letter “Laymen in scientist’s clothing?” (*Nature* 346, 505; 1990). My PhD research on the population genetics of host-plant adaptation of a major horticultural pest interfaces between empirical and fundamental studies and I have come to several conclusions.

Fundamental, theoretical studies tend to be inaccessible to the nonspecialist because of jargon, thus reducing their overall readability, and because of the tendency to use mathematics to explain arguments without attempting to summarize these arguments in a nonmathematical form. (While the importance of mathematics in biology must be recognized, it is for the biologist a tool to aid understanding and not an end in itself.)

Biologists tend to divide themselves into camps of empiricists or theorists, resulting in parallel but non-interacting development. This is mutually disadvantageous as well being bad scientific method (theory and experiment should go hand in hand — few biologists seem to be good at both).

My experience in the United Kingdom was that the teaching of biology at all levels places too much emphasis on learning by rote of theories and facts and pays too little attention to training people to think and work as scientists. I am grateful

that all the science I learned at school was from the Nuffield syllabuses, which placed a strong emphasis on learning ideas from experimental observation and taught me to appreciate the fundamental principles underlying such observations. Such an approach necessarily led to a lesser accumulation of facts and figures, but I believe that it has greatly helped me in my development as a scientist. However, many universities regarded the Nuffield courses as containing too little academic content.

As a young researcher I try to use as many approaches as possible in tackling my research subject, believing that each (empirical and nonempirical) has its own merits and that each, if used properly, complements the other.

If we are to avoid the ‘layman in scientist’s clothing’ syndrome in biology, I believe that we need to educate students in a way that trains them to be scientists. Such training should ensure that they are sufficiently literate to be capable of expressing their ideas effectively in everyday language.

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Investment in UK universities

SIR—The report of Peter Aldhous (*Nature* 347, 216; 1990) about the setting-up by the Japanese pharmaceutical company EISAI of a neurosciences research centre at University College, London, takes the opportunity to castigate British pharmaceutical companies for not investing in UK universities.

I should like to redress the balance a little. About two-and-a-half years ago, I approached some British and overseas drug companies for support to set up a Centre for Applied Neuropsychobiology at the University of Oxford which would collaborate with the university departments of clinical pharmacology and psychiatry and the MRC Unit of Clinical Pharmacology. Several UK and overseas companies showed interest, and Beecham representatives quickly reached agreement in principle on the setting up of the Oxford University Beecham Centre of Applied Neuropsychology contiguous with the MRC unit and the university department of clinical pharmacology. Negotiations followed between the University of Oxford, the Medical Research Council and Beecham Pharmaceuticals, which resulted in a legal agree-

ment covering all aspects of intellectual rights, royalties, publishing policy, staffing and progress review.

Refurbishment of vacant space in the Radcliffe Infirmary, Oxford, began in June 1989, and the laboratories were completed in October 1989. The centre is now up and running, research is under way, publications have already appeared and the administration is working well. Senior scientists, graduate students and clinical scientists are working in the centre and collaborating with Beecham’s own in-house neuropharmacologists, molecular biologists and medicinal chemists.

There are twelve core scientific staff in the centre with agreed options on a further four. The contract for the centre is for ten years in the first instance, during which the investment is likely to be £7.5 million.

The contract was drawn up and signed before Beecham merged with Smith Kline and the new company, Smith Kline Beecham, has endorsed all the arrangements and funding.

This is a significant investment, and the initiative was wholly British.

D.G. GRAHAME-SMITH

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SIR—Infante and Huszagh¹ rightly complain about the now prevailing phenotype among researchers in biology; they describe it as poorly imaginative, lacking scepticism and creativity, but one that is productive in getting data published. They note that “there seem to be fewer and fewer people able to propose new schemes, or even to question the accepted ones”.

But may it not be that there are as many such people now as previously, but that their naturally limited number is diluted nearly to infinity by the large crowd of ‘kit-users’ now working in biological research? This development was unfortunately brought about by the scientific community itself. By irresponsibly advertising biology as being able promptly to solve any urgent human problem — nutrition, health and so on — if only enough manpower (and money) were put into it, and by the obvious expansion-neurosis prevalent in any human enterprise, many who might have been better placed in other, perhaps more lucrative, jobs were attracted to it.

Sceptical and imaginative individuals do still exist among biologists. Recently, John Cairns and coworkers² and Barry Hall³ set good examples for introducing uncertainty by questioning the accepted view concerning the origin of bacterial mutants (see also ref. 4). The present dogma rests on a famous experiment⁵ which proved that infection of a bacterial population by virulent phages does not induce mutations making bacteria phage-resistant. Only those bacteria already resistant before encountering the phage could survive.

Yet with our present knowledge, we can ask how bacteria could ever have mutated if they were killed by the phages within minutes. What was overlooked when the outcome of the experiment became the basis of a universal dogma is (among other things) the possibility that the recruitment of mechanisms for directed mutagenesis might well need some time, which would be available to a bacterial population struggling for survival in a state of nutritional (and not immediately lethal) distress. Ideas of molecular mechanisms for induction of adaptively beneficial mutations may well be beyond the imagination of the ‘kit generation’.

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Origin and spread of AIDS

SIR—The recent report¹ of the detection of HIV-1 sequences in the tissues of a British sailor who died in Manchester in 1959 highlights again the question of when HIV first infected human beings. The same year is also that of the earliest recorded serum samples (from Zaire) found to contain antibodies against HIV-1 (ref. 2). The earliest case of HIV-1 positive serum outside Africa, reported from Norway³, it was that of a sailor who probably became infected in the mid-1960s and whose wife and third daughter died of what would be diagnosed today as AIDS.

There is now little doubt human AIDS began in Africa. Not only is the disease widely spread in central Africa, but only in Africa are there monkey species naturally infected with lentiviruses related to HIV⁴. Although the first such virus was isolated from a macaque⁵ (and therefore named SIV_{MAC}), that animal was probably infected in captivity with SIV_{NM}, for which the African sooty mangabey monkey (SMM) is the natural host.

It remains a puzzle that the two human viruses known to cause AIDS (HIV-1 and HIV-2, the latter isolated from a West African patient^{7,8}) share only 40 per cent homology while HIV-2 and both strains of SIV share roughly 75 per cent homology⁹, and an extra gene *vpx* which probably arose as a duplication of the *vpr* gene¹⁰. These similarities explain both the serological cross-reactivity of SIV and HIV-2 and the circumstances that HIV-2 was recognized¹¹ after an AIDS patient's serum failed to react against HIV-1.

The first plausible explanation for the origin of AIDS by cross-species transfer is due to Noireau in 1987 (ref. 11). He referred to a book published by Anicent Kashamura¹², a member of the Idjwi tribe of the Lake Kivu region in East Zaire. Kashamura deals with the sexual habits of the people of the large African lakes. Noireau quotes the following sentence "To stimulate a man or a woman and induce them to intense sexual activity, male monkey blood for a man or she-monkey blood for a woman, was directly inoculated in the pubic area and also into the thighs and back." Such practices would constitute an efficient means of trans-species transmission and could be responsible for the emergence of SIV infections of man and thus AIDS.

This led to the suggestion¹³ that SIV_{MAC/NM} could have given rise to HIV-2 infection of man in West Africa and that a distinct monkey virus will eventually be isolated which will be similar to HIV-1. The complete nucleotide sequence of a lentivirus recently isolated from a chimpanzee¹⁴ has indeed revealed what has been described¹⁵ as "the missing link".

Obviously, it is not possible to estimate

when the first person became infected with a monkey lentivirus, but the question of when and how the present epidemic began to spread is more tractable. Extensive European contacts with black Africa go back five centuries, accompanied by the forceful transfer of millions of Africans to the New World. Arabs similarly had contact with West and East Africa over a span of hundreds of years and also engaged in slave traffic. Yet there is no evidence for the existence of HIV in Europe, the Americas or Arabia during the past century or even the first half of this century, which argues strongly that the widespread HIV infection in Africa is a recent event¹⁶.

The wide divergence between isolates of HIV-1 and of HIV-2 can then be explained by continuous mutation and infection by SIV from different species of monkeys. Studies of the spread of adult T-cell leukaemia virus (ATLV/HTLV-1) indirectly bear on this question. ATLV was first shown to be the cause of ATL in Japan¹⁷, which was subsequently shown also to be prevalent in parts of Africa¹⁸. Infection with ATLV is uncommon in the West, where most infected individuals are either Japanese or blacks of African origin. So it is not unreasonable to think that ATLV was brought to the West first with the African slave trade and subsequently by Japanese migration to the Americas (United States and Brazil). Unlike HIV, ATLV is not readily transmissible and has not spread widely in the West, but the finding of ATL and its virus among British blacks indicates two transcontinental transfers — from Africa to the New World and from the West Indies to Britain¹⁹. Although infection with HIV is now prevalent in the West Indies, its absence in immigrants to Britain from the Caribbean 40 years ago supports the notion that the virus started spreading to the West Indies more recently.

All in all, the epidemiological evidence thus points to the spread of HIV infection from Africa since the Second World War. The spread seems to coincide with the widespread introduction of syringes and needles from the West, together with vaccination programmes. Syringes arrived together with antibiotics: as the early generation of antibiotics came only as injectable medicines, the needle and syringe became an inseparable from their therapeutic effect. Even now, injectable medication is the treatment of choice in Africa and other countries.

One can readily then envisage that a handful of individuals in Africa somehow became infected with monkey lentiviruses and subsequently transferred the virus to others. This 'syringe and needle' period coincided with improved communication

and extensive migration in Africa. Thus the virus would have been carried from a few isolated rural endemic areas into urban populations, and thence by sexual contacts overseas.

AIDS readily spreads by syringes as seen in drug users and by recent experience in the Soviet Union and Romania. It is also noteworthy that HIV is not the first monkey virus to infect man: in 1934, Sabin and Wright reported the isolation of herpesvirus simiae (B virus) from the brain of a laboratory worker dead after a bite by an apparently normal macaque rhesus monkey²⁰. Since then, there have been more than a dozen such cases. Herpesvirus simiae seems to be the counterpart, in macaques, to herpes simplex in humans; infection is latent and benign. But, on transfer to man, herpesvirus simiae is fatal, and quickly.

Lentiviruses in the African monkeys which are their natural hosts may be similar — the virus becomes deadly only after transmission to another species, in this case man. The significant difference is that the latency period between infection and disease is longer, about 10 years on the average, providing opportunities for spread before the infected person dies.

That is why the outlook is so gloomy. With such a long latency period for HIV and in the absence of an effective drug or vaccine, the spread of infection seems inexorable. Already AIDS is the leading cause of death among women aged 20-40 in the United States and Western Europe²¹. Unless efforts to halt the spread of AIDS succeed, it can only be a matter of time before AIDS is as prevalent in the rest of the world as it is now in some regions of Africa.

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Making the Universe hang together

A simple explanation why most of the mass in the Universe is not visible unites questions such as the ionization of intergalactic clouds and the age of the Universe in an arresting way.

THE past few months appear to have brought endless disappointments for those hunting for the dark matter that is supposed to give the Universe the appearance of being almost held together. Every few weeks or so, there seems to have been yet another demonstration that neither the hypothetical particles called axions nor those called photinos will quite fill the bill. Yet the faith of the hunters continues to be sustained not just by hope, but by the orthodox interpretation of observations.

The Universe is indeed expanding less quickly than would be inferred from the distribution of visible matter in galaxies and the stars of which they consist, suggesting quantities of unseen mass up to five times as great as that which can be seen. And the motion of stars and other objects (hydrogen clouds, for example) in the galaxies close enough to be observed in detail is often consistent only with a total mass much greater than that which can be seen.

The puzzle of the missing mass is not, of course, the only puzzle over something missing from the Universe that now keeps astrophysicists awake at nights. There is also the problem of the "missing" neutrinos from the Sun, the twenty-year-old discrepancy between the flux of neutrinos expected from the Sun as by-products of nuclear reactions in the core and the flux measured by neutrino detectors on the Earth. The first measurements, at the Homestake gold mine in the United States, revealed a discrepancy of a factor of three to four between the expected and measured fluxes. That dilemma has, if anything, been sharpened by more recent measurements, as Michael Cherry explained in a News and Views article a few weeks ago (*Nature* **347**, 708; 1990).

But now there is a chance that the two problems may be linked. That seems to be one implication of an article by Denis W. Sciama, the cosmologist who boasts affiliations with the Universities of Oxford and Cambridge as well as with the international institutes of Theoretical Physics and of Advanced Studies at Trieste (*Phys. Rev. Lett.* **65**, 2839; 1990). More accurately, Sciama offers neutrinos with mass as candidate constituents of the missing mass. And these are likely to have arisen only by the spontaneous conversion of ordinary electron neutrinos into the cousin neutrinos associated with the μ and τ leptons, the more massive analogues of the electron.

This is not a new idea, as Sciama is careful to acknowledge. That the dark matter may be made of neutrinos with mass has been canvassed on several occasions. Sciama's achievement is that he has found a way of linking together observations of several different kinds with a single and tightly constrained hypothesis about the character of the neutrinos. The theory may be "speculative" as he confesses, but it is also eminently testable. How many astrophysicists can say that of their theories?

The starting point is also unexpected — the density of ionized hydrogen in the Galaxy (which can be estimated from the dispersion of the signals from galactic pulsars) and the ionization of hydrogen in remote regions of the Universe (where the redshift is large), which are recognizable by the Lyman- α radiation they emit. Why is there so much ionized hydrogen so widely distributed?

The simple answer is that the ionization requires a source of ultraviolet radiation. Hydrogen in a galaxy will be ionized by the ultraviolet radiation from the stars that it contains, but Sciama argues that this is not a sufficiently powerful source, and that the Lyman- α radiation of the distant hydrogen clouds is also "difficult to explain". So what else is there? What, for example, if the dark matter of which the missing mass consists is liable to spontaneous decay, with the emission of ultraviolet photons? The μ and τ neutrinos, if sufficiently massive, would fit the bill. It is just a decade since De Rujula and Glashow suggested that it should be possible to recognize particulate missing mass by the homogenous ultraviolet background it would create.

Sciama's objective is to fit his hypothesis that the dark matter consists of neutrinos with such data as there are. His anchor-point is an estimate based on Lyman- α radiation from intergalactic clouds that the flux of ionizing photons must be less than $6 \times 10^5 \text{ cm}^{-2} \text{ s}^{-1}$. As it happens, the density of neutrinos throughout the Universe is believed to be about 100 cm^{-3} , while the energy required to ionize a hydrogen atom in its own rest-frame is 13.6 eV.

There are a very few straws with which to make bricks. Sciama first supposes that the ionizing photons arise from the conversion of one particle (with mass m_1 , say) into another (with mass m_2). If m_1 is substantially larger than m_2 , then the energy of the photon is simply $\frac{1}{2}m_1$. (Without

approximation, it amounts to $(m_1^2 - m_2^2)/2m_1$.) This in turn fixes the mass of the more massive of the two neutrinos as having to be greater than 27.2 eV. Taking account of a recent failure to observe decay photons from the Coma galactic cluster (with less energy than that required for hydrogen ionization), and allowing for the recession of the cluster calculated from its redshift, he reaches the more stringent limit that $m_2 > 13.9 \text{ eV}$.

But that is only a lower limit. How far above it does the true mass lie? Or how to obtain an upper limit for the greater of the two masses? Ingeniously, Sciama links together in two steps almost everything there is in cosmology. First, he obtains a more accurate neutrino density ($115 \pm 2 \text{ cm}^{-3}$) by using the measured intensity of the microwave background and some lore derived from the standard model of particle physics. Then he notes that the ionizing flux will be determined by the proportion of these neutrinos not receding so quickly that the energy of the photons they emit is reduced below the ionization threshold, which brings Hubble's constant (which scales the expansion speed) into the argument.

Two assumptions then make everything hang together. First, Hubble's constant is about 50 km s^{-1} per megaparsec (near the lower limit of the range in which it is constrained by distance measurements) and the lifetime of the more massive neutrino is less than 3×10^{23} seconds. Then, the mass of the heavier neutrino m_1 lies between 27.8 and 30 eV, while that of the less massive neutrino (either the electron or the τ neutrino) is less than 3 eV. And, surprising though it may seem, the mass density of neutrinos throughout the Universe turns out to be more than 20 times greater than the estimated density of baryonic matter. Plainly, there is enough missing mass to satisfy everybody. For what it is worth, the age of the Universe also tumbles out at 12,000 million years.

What does all this mean? It would be easy but mistaken to dismiss the argument as a tissue of speculation. In reality, Sciama has a reputation among cosmologists for believing that the numbers matter, even if there too few of them for comfort. What he has done is to construct a picture of the Universe that has the merit of being testable. It will be interesting to see what they make of it at the symposium on cosmology and particle physics arranged for next week at the University of Sussex.

John Maddox

The seismic cycle pursued

Allan G. Lindh

In a careful analysis of the seismicity of the past 140 years in the San Francisco Bay area, on page 595 of this issue¹, Sykes and Jaume argue that three large earthquakes were each preceded by 10–20 years of increased seismicity in the surrounding region. In principle, this kind of phenomenon might contribute to improving the precision of intermediate-term earthquake predictions to a decade or less, although first more must be learned about the generality and specificity of the phenomenon.

The quest for such seismic patterns goes back a long way. In his pioneering 1923 discussion of Californian seismicity², Bailey Willis commented on the same periods of increased seismicity in the San Francisco Bay area: "in the northern coast ranges there was a group of earthquakes of high severity occurring about 1840, another between 1860 and 1870, and again between 1887 and 1906". Even more remarkable is his observation that "in southern California there were several very severe earthquakes between 1852 and 1857". These are the five years leading up to the great 1857 (magnitude 8.25) earthquake on the southern San Andreas. It is clear that Willis believed "that earthquakes succeed each other in groups with intervals of relative quiescence between each group." He did not comment on the fact that each period of activity culminated in a large event, although it is hard to believe he had not noticed the pattern.

Also in 1923, on the opposite side of the Pacific, Tokyo was destroyed by a great earthquake and fire³. A Japanese seismologist, Akitune Imamura, had correctly anticipated this event. His successful prediction was based on a careful analysis of historic seismicity, an early form of what we would call today a seismic-gap theory, supplemented by levelling observations of tilting of the adjacent coastline. Subsequently he noted that "the 1923 earthquake occurred after 150 years of repose followed by 70 years of activity, during which time about a dozen local destructive shocks were felt"⁴, and that similar patterns were observed before the 1707 Kwantō earthquake.

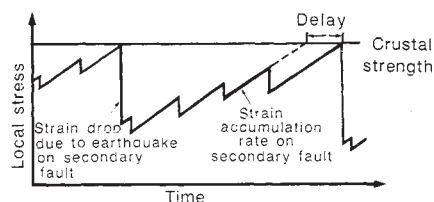
Following the 1957 San Francisco earthquake (magnitude 5.3), Don Tocher⁵ reanalysed the historic data from the area, noting the near total absence of seismicity from 1911 to 1955 and that the dormancy following the 1906 earthquake had ended with the three magnitude-5-plus events from 1955 to 1957. In an analysis of the seismicity of the Kamchatka Peninsula and Kurile Islands^{6,7}, S. A. Fedotov correctly predicted, in a qualitative long-term sense, four large earthquakes in the

northwest Pacific in the late 1960s and early 1970s. Analysing the patterns of regional seismicity accompanying the cycle of strain accumulation and release in great earthquakes, Fedotov coined the term seismic cycle to describe the modulation of seismicity. His description of the last active stage of the cycle — "approximately 15 years before the next disastrous earthquake, the seismic activity begins to increase"⁷ — is remarkably similar to the conclusions of Sykes and Jaume in this issue, although the data could hardly come from a more dissimilar tectonic setting.

Kiyoo Mogi's reanalysis of Japanese seismicity in terms of seismic gaps and recurrence intervals took the notion of precursory seismicity increases one step farther by showing in some detail that the last 20 years of seismicity preceding the 1944 and 1946 Nankaidō earthquakes was located in a halo pattern — the 'Mogi doughnut' — around the portion of the fault about to fail⁸.

But despite the parallels between these reports, and although some are so compelling that they can stand alone, there are important differences. Imamura and Tocher describe broad regional seismicity increases on a timescale of 50–70 years whereas Fedotov, Mogi and Sykes and Jaume are concerned with timescales of 10–20 years. Indeed, the phenomenon probably contains a large stochastic element, dependent in part on geological and tectonic differences between regions, and it is unlikely that any simple universal pattern will be found. Nevertheless, the fact that recurrent plate-boundary earthquakes are the product of large-scale crustal processes of strain-energy accumulation and release^{9,10} makes it intuitively appealing that there should be some seismic manifestation of the higher stresses that must accompany the final stages of the strain accumulation process.

One class of models to explain the seismic cycle is represented by the suggestion of Kunihiko Shimazaki that the increased seismicity of the late stages of



Hypothetical change in the local stress with time on a secondary fault. There is a delay (shown) in the earthquake on the secondary fault owing to a drop in strain due to a great earthquake on a nearby primary fault. (Adapted from ref. 12.)

the seismic cycle is a simple consequence of the accumulation and release of strain within the brittle crust^{11,12}. Each secondary fault in the region is hypothesized to have its own strain accumulation and release cycle, albeit of a smaller scale and lower rate than the main cycle. When a great earthquake occurs on the primary fault in the region — the San Andreas in the case of the San Francisco Bay region — it releases strain across the secondary faults (see figure) to a degree depending on their distance from the primary fault. The overall effect throughout the region would be to delay the seismic cycles on the secondary faults, and thus the apparent increase in regional activity at the end of the main cycle actually marks the end of this regional suppression that has lasted since the beginning of the cycle. The model has the advantage of being sufficiently specific that detailed simulations could be attempted to test its consequences against the observed spatial and temporal patterns of seismicity. But in terms of Sykes and Jaume's report, it has the disadvantage that, in its simplest form, it does not predict a short-term acceleration in activity at the very end of the cycle.

A high-precision down-hole strain record from near the recent Loma Prieta earthquake (magnitude 7.1) led Mike Gladwin to suggest that that event was preceded by about 18 months of accelerating slip on the portion of the plate boundary beneath the Loma Prieta rupture zone — that is below 15–20 km depth¹³. This is consistent with the fact that the Loma Prieta earthquake was preceded by two unusual magnitude-5 earthquakes, in June 1988 and August 1989, at the north end of the Loma Prieta segment, where no such events had occurred since 1906. They were located at about 15 km depth, which puts them among the deepest events recorded on this segment before the Loma Prieta event.

Deep premonitory slip could, in principle, account for increased regional seismicity at the end of the seismic cycle by accelerating the strain accumulation rate on regional faults. But in fairness, no definitive geodetic observations of accelerating strain rates have yet been made.

The truth is that on the basis of seismicity observations alone, it will be hard to distinguish between competing models for the seismic cycle, even with models as contrasting as Shimazaki's and Gladwin's which have significantly different implications for efforts to detect the final stage of the cycle. But it is now possible to deploy down-hole strain meters with a usable sensitivity of 1 part in 10^9 over periods of a few minutes to a few weeks, and 1 part in 10^8 for periods as long as 1–2 years. Such arrays are now deployed as part of the Parkfield Prediction Experiment, along the San Andreas in central California, and in the Tokai Gap Prediction Program

in the Tokai Gap Prediction Program south of Tokyo, where an extraordinary effort is being made to predict the next great earthquake at the north end of the Nankai trough.

In addition, the development of the Global Positioning Satellite (GPS) system for geodetic monitoring now allows dense geodetic observations to a precision approaching 1 part in 10^7 . Although lacking the extreme sensitivity of the down-hole strain meters, GPS has the advantage of long-term stability, and is deployable in large, dense arrays. In particular, broad arrays spanning the San Andreas system may elucidate the motion of the deep plate boundary.

The fundamental difficulty in trying to unravel seismicity patterns is that although earthquakes give by far the highest-resolution information on deep fault processes, they are a very nonlinear product of those processes. Until we understand the spatial pattern and history of deep plate motion, how the strain energy is transferred upwards into the brittle seismogenic crust, and how that

strain energy is released as earthquakes, much of what we do in this field will remain in the realm of barely constrained speculation. The San Francisco Bay area provides a unique opportunity to acquire the diverse data needed to reduce the element of speculation. □

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IMMUNOLOGY

The invariant dating service

Frances M. Brodsky

CLASS I and class II major histocompatibility complex (MHC) molecules bind antigenic peptides from different intracellular sources and therefore have distinct immunological functions. This difference in peptide acquisition is attributable to the transient intracellular association of the 'invariant chain' with class II molecules^{1,2}. The invariant chain polypeptide forms a complex with class II α and β chains during their biosynthesis and export, which dissociates before class II expression on the cell surface. Class I molecules lack an equivalent intracellular companion. It has been proposed that the invariant chain could determine the intracellular site of peptide acquisition through interaction with the binding site, by acting as a chaperone and/or by directing transport of class II molecules as an escort. Now, on page 600 of this issue³, Lotteau *et al.* describe evidence for a transport function.

The authors report that the invariant chain influences the intracellular localization of class II molecules and, in agreement with a paper by Bakke and Dobberstein⁴ in *Cell*, they demonstrate that its cytoplasmic domain has intracellular targeting signals. The formation of the invariant chain/class II complex prevents class II molecules from binding peptides^{5,6}; but subsequent direction by the invariant chain of bound class II molecules to an intracellular compartment, where the two can dissociate, could allow the class II

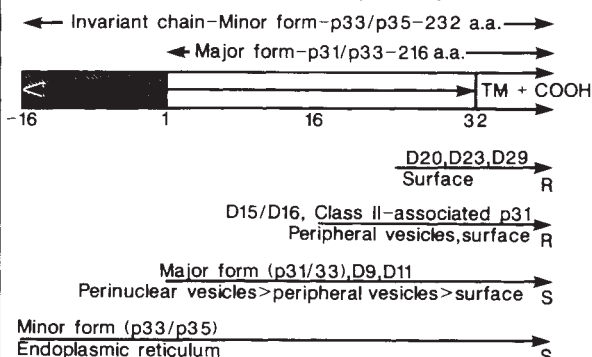
molecules to be exposed to and bind a different pool of peptides from those acquired by class I molecules. So it may be the split personality of the invariant chain as both a chaperone and an escort service that contributes to differentiation of function of class I and II MHC molecules.

Class II molecules can bind peptides derived from internalized and degraded exogenous antigens, and present them to helper T cells⁷. These peptides are not presented by class I molecules, which during their biosynthetic assembly, bind peptides produced from endogenously

synthesized viral proteins⁸, and stimulate cytotoxic T cells. Class I, unlike class II molecules, do not come into contact with endocytic compartments during their export from the cell⁹. Conversely, the association of the invariant chain with class II molecules in the endoplasmic reticulum prevents binding of peptides that are bound by class I molecules. Dissociation of the invariant chain makes the class II peptide-binding site accessible^{5,6} and requires low pH and proteolytic activity, typical of the endocytic pathway.

The missing link in this scheme has been the mechanism by which class II molecules and the associated invariant chain are targeted to the endocytic pathway^{9,10}, which is an unusual route for export of constitutively expressed membrane proteins. Lotteau *et al.*³ demonstrate that, in HeLa cells, targeting of transfected class II molecules to intracellular vesicles requires cotransfection with the invariant chain. Expressed in the absence of the invariant chain, class II molecules are found primarily at the cell surface. Thus, in antigen-presenting cells that express both molecules, the invariant chain could account for the difference in intracellular location of class I and class II molecules.

The identification of intracellular targeting signals on the invariant chain^{3,4} (see figure), suggests that the signals determine the intracellular location of the class II/invariant chain complex. By analysing transfected invariant chains and invariant-chain deletion mutants, Lotteau *et al.* and Bakke and Dobberstein demonstrate that the cytoplasmic domain (N terminus) of the major wild-type form of the invariant chain has a signal that directs the chain to endocytic intracellular compartments (see figure). Removal of this signal results in cell-surface expression of invariant chains. Without coexpression of class II molecules, a more distal signal that prevents maturation and export of the invariant chain seems to dominate. The



intracellular destinations of transfected invariant chains and deletion mutants. The intracellular locations of the wild-type and mutant invariant chains transfected into HeLa cells³ or CV1 cells⁴ are indicated below each arrow, which begin at the most distal residue where the intracellular location was observed. The target signals for localization must be between the start sites of the arrows. The dominant wild-type form of the invariant chain (216 amino acids) is designated p31 (ref. 3) or p33 (ref. 4). A secondary form (232 amino acids, p33 or p35) is produced by initiation of translation from an upstream start site. Deletion mutants are indicated by D, together with the number of N-terminal residues removed from the cytoplasmic domain of p31. The D15 mutant tested in CV1 cells has the same sequence as the D16 mutant tested in HeLa cells (both start with methionine at position 16), although they are designated differently. Their localization patterns differ in CV1 and HeLa cells. The letters S and R at the right of each arrow show the dominant pattern of endoglycosidase H sensitivity (S) or resistance (R) for each form of transfected invariant chain. The same constructs expressed in CV1 cells or HeLa cells differ in degrees of sensitivity to endo H, but the dominant pattern is the same.

Trapped exons

A difficulty in isolating genetic loci by 'reverse genetics' is how to identify the gene of interest from the surrounding DNA. A new technique described by G. Duyk and colleagues, called exon trapping, allows the direct recovery of transcribed DNA sequences (*Proc. natn. Acad. Sci. U.S.A.* **87**, 8995–8999; 1990). The DNA is cloned into a retroviral vector, downstream of a splice-donor site and an intron that contains a portion of the β -galactosidase gene. DNA fragments which contain a splice-acceptor site can generate spliced RNA, which after packaging can be identified because of the loss of β -galactosidase activity. Although the method is time-consuming and cannot detect genes without introns, it should become a powerful weapon in the molecular geneticist's arsenal.

Hard stuff

IN trying to understand how diamond films can be grown at low pressure, researchers at the Ford Motor Company may have stumbled on a crystal that could harder still than diamond. Using a reliable semi-empirical formula, M.A. Tamor and K.C. Hass (*J. Mater. Sci.* **5**, 2273–2276; 1990) calculated the lattice parameters for a structure which they term H6 and which can be likened to graphite with one bond in each planar hexagon twisted by 60° to bind covalently to the next plane. The structure is less dense than diamond, as befits a low-pressure allotrope, and metallic, as the surfaces of growing diamond films appear to be. Moreover, as required, it can be deformed continuously to give the diamond structure. But it remains to be seen whether the suggestion that the structure is 50 per cent harder than diamond will be borne out by more detailed calculations.

The right size

MANY vertebrate lineages show a tendency to extreme smallness that imposes constraints on their anatomy. In the Palaeozoic amphibian *Quasicaecilia texana* from the Permian of Texas, discussed by R. L. Carroll (*Palaeontology* **33**, 893–909, 1990), the back of the skull is dominated by an enormous otic (inner ear) capsule that forces the jaw articulation forwards, with consequent alteration of jaw musculature and, presumably, lifestyle and diet. The 15-mm-long skull of *Quasicaecilia* is by no means the smallest: modern salamanders of the genus *Thorius* can have skulls as tiny as 3.3 mm long, of which the eye sockets occupy a relatively enormous 27 per cent. The enigmatic caecilians, burrowing worm-like amphibians, have skulls very similar to *Quasicaecilia* — hence the name — but the similarity may be largely due to convergent responses to small size rather than common ancestry.

minor wild-type form of the invariant chain, with an extended N terminus due to the use of an alternative site of translation initiation, has an additional endoplasmic-reticulum retention signal that dominates other signals³.

Using immunoelectron microscopy, Bakke and Dobberstein⁴ found intracellular forms of the invariant chain in both early and late endosomes. Lotteau *et al.*³ propose that the perinuclear vesicles to which the main wild-type form of invariant chain localizes in the absence of class II molecules are autophagosomes, because they are characterized by a lysosomal marker but are reached directly from the endoplasmic reticulum. They find that coexpression of class II molecules and the invariant chain results in the targeting of both molecules to a different population of more peripheral intracellular vesicles with endosomal characteristics. Class II α and β chains must obscure the perinuclear signal and allow the peripheral vesicle targeting signal to dominate. Coexpression of the α chain alone with the invariant chain results in α chain localizing with each form of invariant chain, confirming that the invariant chain can influence the transport of class II molecule subunits and that it must also be combined with the β chain for selection of its secondary intracellular targeting signal. Class II α and β chains could influence transport of the invariant chain with signals of their own, although the invariant chain alone has signals capable of targeting the complex.

Procedures

The slight differences between the behaviour of invariant chains introduced into HeLa cells (Lotteau *et al.*) and CV1 cells (Bakke and Dobberstein) can be attributed to variation in experimental procedures, and to presumable differences between the transport pathways in the transfected cell types. It is notable that both HeLa cells and CV1 cells, which do not normally express class II molecules or invariant chains, have the recognition machinery to respond to the transport signals of these molecules. Data from Salamero *et al.*¹¹ suggest that mouse L cells are not capable of this recognition. In L-cell transfectants, the presence or absence of the invariant chain does not seem to influence the degree to which the class II molecules intercept the endocytic pathway.

Although the invariant chain can influence the intracellular transport of class II molecules, it is not required for the export of class II molecules from the endoplasmic reticulum¹², nor is it absolutely required for class II presentation of antigenic peptides derived from exogenous proteins¹³. It is also not required for class II presentation of peptides from endogenous antigens¹², which may be

acquired by class II molecules from the class I peptide pool, provided that the invariant chain is not bound. In addition, class II molecules can gain access to the endocytic pathway by endocytosis after surface expression and may be able to exchange bound peptides in the endosome¹⁴. Given these options for class II molecules, why is the invariant chain needed at all? And how can its presence influence antigen presentation with so many alternative modes of peptide binding for class II molecules?

Influence

The invariant chain's chaperone-like qualities, so unusual for an escort service, may account for its further influence on the function of class II molecules. The synthesis of class II molecules in the absence of the invariant chain alters the folding of the peptide-binding domains¹⁵, so that the binding ability of some peptides is enhanced and that of others is lost¹⁵. In helping to fold the antigen-binding site, the invariant chain should enable class II molecules to bind to a wider range of antigenic peptides while blocking peptide binding in the endoplasmic reticulum. The chain's targeting to the endocytic pathway and its removal, which occurs under conditions that also generate antigenic peptides, allows class II molecules to acquire these peptides directly rather than relying on exchange. So where a particular antigenic peptide is generated and how well it can bind to a class II molecule will determine whether its presentation requires the invariant chain. In turn, the invariant chain increases the versatility of class II molecules, maximizing their exposure to exogenous antigen and allowing them to have roles different from those of class I molecules. Perhaps the best way to think of the invariant chain is neither as a chaperone nor an escort, but rather as a dating service which provides a wide range of pre-selected partners but does not restrict which of them you take home. □

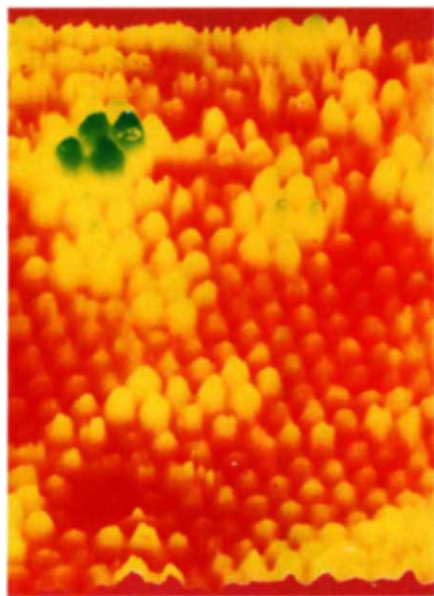
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Chemistry comes round

Philip Ball

WHEN in 1987 physicists at a meeting of the American Physical Society held an all-night session to discuss the newly discovered high-temperature superconductors, they may have established a precedent. Certainly this comparison was on the tongues of some of the 500-plus participants of the post-midnight symposium staged by the Materials Research Society at its fall meeting*, who had gathered to hear the latest news on the sixty-carbon molecule dubbed buckminsterfullerene. But although it is tipped to provide new directions in organic, inclusion and polymer chemistry, lubrication and perhaps electrochemistry and semiconductor



Buckminsterfullerene (C_{60}) molecules packed in a hexagonal monolayer on a gold surface. The image is obtained with a scanning tunneling microscope (STM), each bump representing a single molecule. The reason for the variation in height is not clear, but possibly the taller species are the slightly elongated C_{70} molecules. Resolution of individual carbon atoms is not yet possible. These images, obtained by Donald Bethune *et al.*, and related results by J.L. Wragg *et al.* are discussed on pages 621 and 623.

technology, the jury remains out on whether this promise will be fulfilled.

The discovery, by a collaboration between laboratories at the University of Arizona and the Max-Planck-Institut at Heidelberg, of a means to isolate the football-shaped C_{60} molecule in solid form (W. Krätschmer *et al.* *Nature* **347**, 354–358; 1990) undoubtedly broadens the horizons of carbon chemistry: in the words of Harry Kroto (Sussex), one of the fathers of C_{60} research, "round carbon is in".

Two factors lend particular drama to

the discovery. The first is that, although it is five years since the molecule was proposed as a possible component of interstellar dust after showing up in mass spectra of carbon soot (H. W. Kroto *et al.* *Nature* **318**, 162–163; 1985), the independent efforts of several groups to prepare a pure, solid form began to converge within a few months of each other earlier this year. Kroto's group had already obtained the red solvent-extracted C_{60} solutions when the *Nature* paper appeared, and a team from IBM Almaden had at that time several papers in press elsewhere reporting their own synthetic route.

The second factor is that the material turns out to be astonishingly easy to make. All one needs, according to Richard Smalley of Rice University, is a commercial arc HAEMOGLOBIN

welder, a standard vacuum line and some graphite rods. Such simplicity has allowed many laboratories rapidly to duplicate the initial results. But this kind of crude cookery runs against the grain for synthetic chemists, who, as Robert Whetten (University of California at Los Angeles) explained, had been searching in vain for elegant synthetic routes to the C_{60} cage.

Surprisingly little attention seems to have been devoted so far to exploring the "round organic chemistry" that Kroto predicts, although James Heath at the University of California at Berkeley reported success in osmylating the unsaturated framework. Osmylation is a standard precursory step in the introduction of functionality to unsaturated hydrocarbons, and Heath suggests that it could lead to carbon-carbon bond cleavage, opening a hole in the cage through which one might insert guest atoms. □

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Molecular inventiveness

M. F. Perutz

THE haemoglobins of all bony vertebrates are tetramers made up of a pair of closely, but not covalently, linked $\alpha\beta$ dimers. Judging by the sequence similarity of the residues in contact with the haems and with neighbouring subunits, all their tertiary structures are similar to that of sperm-whale myoglobin and they all share a common allosteric mechanism based on a transition between the same two quaternary structures.

Haemoglobins of invertebrates, plants, fungi and bacteria also share tertiary structures similar to that of myoglobin, but they include monomers, dimers, oligomers and polymers assembled in many different ways¹. In the dimeric haemoglobin of the blood clam *Sapharca inaequivalvis*, the haems and the haem-linked helices E and F of neighbouring subunits are in contact rather than facing outwards as in vertebrate haemoglobins^{2,4}. In the tetrameric haemoglobin of the 'fat innkeeper' worm *Urechis caupo*, the helices B and D and the GH corner make contact around one twofold symmetry axis, and helix E and the AB corner make contact around the other, whereas in vertebrate haemoglobins all these segments face outwards⁵. In the polymeric haemoglobin of the earthworm *Lumbricus terrestris* three different globin chains are linked by disulphide bridges, one of them between cysteines in helix A and the other between cysteines in the neighbouring GH corner⁶; both of these segments face outwards in vertebrate haemoglobins. These haemoglobins are all made up of globin chains of a relative molecular mass of about 16,000

(M_r 16K), the same as vertebrate haemoglobins, but the subunit contacts are different from those in vertebrates and from each other.

The haemoglobin of the brine shrimp *Artemia* is a dimer made up of α and β chains of $M_r \sim 130K$ (ref. 7). On page 653 of this issue⁸, Manning *et al.* show that these chains consist of homologous, myoglobin-like domains linked in tandem.

Domain 4 of *Artemia* haemoglobin: residues at invariant non-polar internal sites.

A8	Ile	E15	Phe
A11	Ser	E18	Leu
A12	Trp	E19	Leu
A15	Ala	F4	Leu
B9	Leu	F5	Gly
B10	Phe	G5	Phe
B13	Met	G13	Ile
B14	Phe	G15	Ile
C4	Tyr	G16	Leu
CD1	Phe	H8	Trp
E4	Phe	H11	Phe
E8	Met	H12	Phe
E11	Val	H15	Ser
E12	Leu	H19	Ile

- Trp A12 Important spacer between helices A and E.
 Lys A14 Found in many globins.
 Arg B12 Found in many globins.
 Pro C2 Determines BC corner.
 Phe CD1 Haem contact.
 Phe CD4 This is Phe CD6 in *Artemia* owing to insertion of two residues in CD.
 His E7 Distal histidine.
 Val E11 Distal valine.
 Leu F4 Haem contact.
 His F8 Haem contact.
 Phe G5 Haem contact.
 Lys H10 Found by Bashford *et al.*¹¹ in many globins.
 Tyr H8 Internal spacer. This is Trp in *Artemia* as in human γ -globin.
 Leu H19 Haem contact. Ile in *Artemia*.

*Materials Research Society Fall Meeting, Boston, 26 November – 1 December 1990.

Moens *et al.* had already determined the amino-acid sequence of one of the domains and found that it contained residues in key positions characteristic of the globin fold⁹. Manning *et al.* isolated several DNAs complementary to *Artemia* haemoglobin messenger RNA, cloned them and sequenced the largest of them. They found nine globin-like domains connected by peptide linkers, including one domain with the sequence determined by Moens *et al.* None of the domains has a sequence similarity greater than 27 per cent with any other globin, but their structural similarities with the globins are unmistakable all the same. Kendrew, Watson and I discovered many years ago that globins show an almost invariant pattern of hydrophobic residues at internal sites; occasionally one or the other of these sites is occupied by a serine or a threonine¹⁰. Fourteen residues fulfilling important functions are also conserved in many globins. The table confirms that both of these criteria are satisfied in one typical domain (T4) of *Artemia* haemoglobin.

Manning *et al.* report that a few polar side chains occur at 'forbidden' internal sites. One is a glutamate at G15 in domains 1 and 5. The atomic model of human haemoglobins shows that this could be compensated by Arg G19 in domain 1 and by either Arg B8 or Lys B12 in domain 5. In three of the domains, polar residues also occur at E14. Following Bashford *et al.*¹¹, the authors refer to this as an internal site, but in fact my model shows that it lies on the surface and we also listed it as such in 1965 (ref. 10). In summary, none of the nine domains contains an uncompensated polar side chain that would be incompatible with the globin fold. Prolines do occur at G14, five residues from the end of helix G, in three of the domains, which implies that these helices are kinked, but such kinks could be accommodated in the globin fold.

The nine domains are connected by

eight linkers, each containing 11–14 residues. Six of the linkers contain prolines located five residues from the C termini, as if providing essential kinks. Each of the linkers contain between three and five polar residues, which implies that the linkers must be in contact with water.

What could be the quaternary structure of *Artemia* haemoglobin? Electron micrographs show that it is a cylinder 120 Å wide and 70 Å thick¹². The external dimensions of myoglobin are 45×35×25 Å (ref. 13). If we take 35 Å as its average diameter, then a ring of nine myoglobin-like domains in close contact would describe a circle of 100 Å diameter, consistent with an external circumference of about 120 Å. Two such rings, one α and β chain, placed one above the other, would form a cylinder 70 Å high. It is intriguing that there is a rudiment of a linker, including the proline, attached to the C terminus of the last domain, as if designed to form a salt bridge with the N terminus of the first domain, so the ring's head can bite its tail.

The haemoglobin of the 'fat innkeeper' worm shows neither cooperativity of oxygen binding nor a Bohr effect, whereas the haemoglobins of the brine shrimp

Artemia, the blood clam *Sapharca* and the earthworm exhibit both^{1,14–16}. In vertebrate haemoglobins, cooperativity arises from transitions between two alternative quaternary structures which are triggered by a movement of the iron atom and the iron-linked histidine relative to the porphyrin. Comparison of the structures of the deoxy- and carbonmonoxyhaemoglobins of *Sapharca* has revealed the same movements, but the stereochemical mechanism of their transmission to the neighbouring subunit is quite different from that in vertebrate haemoglobins, because the subunit contacts are different^{3,4}. The earthworm and the brine-shrimp haemoglobins each have distinct quaternary structures with different subunit contacts, so that each of these species must have evolved different ways of transmission across these contacts in order to achieve cooperativity of oxygen binding and a Bohr effect. This illustrates once again the astounding inventiveness of nature even on the molecular scale. □

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SEMICONDUCTOR DEVICES

Chaos in quantum billiards

P. C. Main

THE 'billiards stadium' model of quantum chaos, the model most accessible to theoretical treatment, could be recreated in an experimental version, according to new work¹. Loosely stated, a ball is confined to move, without friction, on an oval billiard table. Depending on the precise shape of the table, the motion of the ball is either ordered or chaotic. Although it is possible to perform 'experiments' on such systems by computer simulation it is not so easy to construct a real device to study the phenomenon. But Jalabert *et al.* now suggest¹ that billiards stadia could be fabricated using modern techniques of two-dimensional semiconductor devices and that the quantum chaos could be revealed in the paths through them followed by electrons.

Although classical chaos, in which the purely deterministic laws of newtonian mechanics can give rise to completely unpredictable outcomes, has been thoroughly investigated, its quantum mechanical analogue, involving the non-deterministic Schrödinger equation, has not. The favoured theoretical model, the billiards stadium, is used for comparing the two cases because the equations can be readily solved in each. But practical experiments to test the solutions have been harder to devise. In resorting to layered semiconductor devices, or 'heterostructures', Jalabert *et al.* propose to build on

the success of solid-state electronics in probing several unusual macroscopic quantum phenomena.

The advantages of heterostructures are twofold. First, the electrical properties are entirely determined by the most energetic electrons (those at the so-called 'Fermi' surface in the momentum distribution) all of which travel with the same speed in a two-dimensional plane. Second, modern fabrication techniques enable

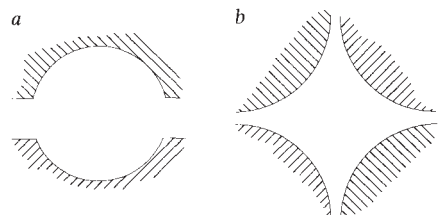


FIG. 1 The two devices considered by Jalabert *et al.* for studying chaotic dynamics. The electrons may only move in the unshaded regions, being reflected elastically from the walls, after injection from one of the contacts.

devices smaller than 1 μm across to be constructed in material for which the electrons' mean distance between elastic collisions exceeds 10 μm, so that their motion is ballistic. In the two structures Jalabert *et al.* analyse (Fig. 1), electrons are injected into the 'stadium' from, say, the left-hand contact and, in general, will rattle around, undergoing many collisions with the walls

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before finally disappearing into one of the contacts.

An earlier theoretical study² on the structure in Fig. 1a did show all the ingredients of a classically chaotic system. The incident electrons were described by their positions and angular direction in the injection contact, rather as one might describe a ray of light. For some values of these parameters, the final state of the electron (the lead into which it finally emerges) is chaotic. The usual signs of chaos — fractal behaviour and self-similarity — emerged in the analysis.

It turns out that the electron trajectories that are important for the chaotic motion involve a large number of collisions within the stadium. Indeed there are some trajectories that are totally confined and never emerge. But that also means that they are inaccessible to the injected electrons. Nevertheless these trapped orbits are the key to the chaotic motion (the 'strange repeller') and the real trajectories lie arbitrarily close to them. Generally, the narrower the contacts relative to the radius of the stadium the more likely the motion is to be chaotic.

The question addressed by Jalabert *et al.* is: how might this chaotic motion be observed? It is straightforward to construct structures such as those shown in Fig. 1 and measure their electrical properties. But these properties are determined by the total transmission probabilities of electrons between the various contacts. The chaotic motion, on the other hand, exists at the level of individual electron trajectories and so would seem to be unobservable by electrical measurements.

The authors point out that although the trajectories are classically chaotic, the electrons themselves are quantum particles. This means that quantum interference can occur between the different classical orbits. One way to think of this is that the quantum electron behaves as a set of partial waves following the classical trajectories. Where these cross, interference will occur, the phase difference between the waves depending on the path difference. The application of a magnetic field can alter these phase differences to an extent that depends on the amount of magnetic flux enclosed by the loop formed by the two trajectories. In a given magnetic field this flux is determined by the area enclosed by the loop. Varying the magnetic field changes the interference properties of the system. This feeds through into the total transmission coefficients and leads to aperiodic fluctuations in the resistance of the structure as the magnetic field is changed (Fig. 2). Similar fluctuations in magnetoresistance of disordered conductors have been known and understood for some time³.

What are the chances of observing the effects described? Numerous experiments⁴ have already been carried out on

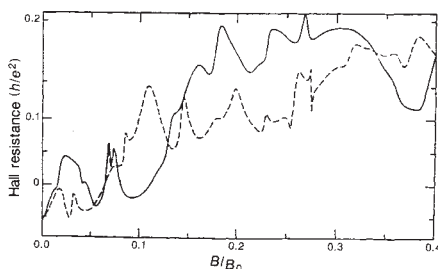


FIG. 2 Chaos in a semiconductor billiard stadium should be apparent from fluctuations in its 'Hall' resistance as a function of the applied magnetic field B/B_0 , calculated here for the structure in Fig. 1a for two slightly different values of the electrons' 'Fermi' velocity. (After ref. 1.)

devices similar to those shown in Fig. 1, and aperiodic fluctuations have even been seen in the magnetoresistance and Hall resistance in some cases. The problem is that any set of electron trajectories can give rise to interference effects, as Jalabert *et al.* themselves admit. But the authors argue that the chaotic set has a particular universal signature which can be obtained, for example, by Fourier analysis of the resistance fluctuations. Other non-chaotic stadia give rise to non-universal behaviour but it is clearly going

GENETICS

Evolution of the X chromosome

Mary F. Lyon

OHNO'S law¹ of the constancy of the genetic material of the mammalian X chromosome has been accepted for over 20 years. It has now been challenged by work of Watson *et al.*, published in *Proceedings of the National Academy of Sciences*², on the X chromosome of a monotreme, the platypus.

The rationale of Ohno's law is that the organism is adapted to the effectively haploid dose of gene products of X-linked genes resulting from X inactivation (the silencing of one X chromosome in females to provide equal dosages of X-linked gene products in males and females), and that translocation of these genes to an autosome would result in inviability. There are no known exceptions to this law among over 20 species of placental mammals from several orders. In marsupials also, genes from the human X long arm (Xq) are X linked but genes from the human X short arm (Xp) are autosomal³. Thus the marsupial X appears to have been 'frozen' at a stage when it was smaller, and constitutes 3 per cent of the genome in contrast to 5 per cent in placental mammals^{4,5}. Wrigley and Graves⁶ have suggested that monotremes show an early form of X-inactivation in which only the relatively small differential segment of the short arm undergoes inactivation, and only in some tissues. The long arm of the platypus X

to be very difficult to provide definite experimental proof that chaos has been observed. The results in Fig. 2 are calculated at zero temperature assuming the electrons to be infinitely coherent. Another problem is that phase coherence is lost, usually by collisions with other electrons, over the inelastic scattering length. When the length of a typical chaotic trajectory is greater than this length, the resistance fluctuations will no longer be visible. This implies that for the best observation of the effect, very small structures are required operating at very low temperatures, much less than 1 K. Nevertheless, I shall be surprised if we do not see at least one paper in the next few months claiming the observation of quantum chaos in devices like those described by Jalabert and colleagues □

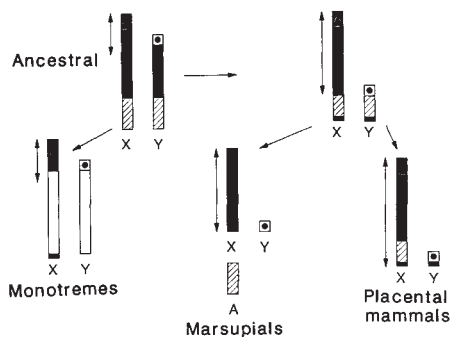
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seems to be homologous with the Y and shows no sign of inactivation.

Watson *et al.*² have now mapped eight genes from human Xq to the platypus X chromosome. Five mapped to the differential segment of the short arm, whereas the remaining three (GDX, P3 and G6PD) mapped to a putatively Y-homologous and non-inactivated region at the distal end of the long arm. Watson *et al.* therefore suggest that the presence of these three genes on the long arm of the platypus X pre-dates inactivation, and that the conservation of mammalian X chromosomes may in fact not be a consequence of X inactivation, but rather a result of chance.

In support of this idea, the authors cite the apparent conservation intact of certain autosomes between human and cat. However, the most detailed evidence on comparative gene mapping comes from human and mouse. In both species around 1,500–2,000 gene loci have been mapped, and the positions of over 500 homologous loci are known in both species⁷. The number of human chromosomes with which any mouse chromosome has homologies ranges on the autosomes from two to seven — that is, no mouse autosome is represented intact on a single human chromosome. As homologies of only half of the map are so far known, the numbers



Genetic content of X chromosomes of present mammalian subclasses and possible ancestral chromosomes. Material from human Xq, stippled; human Xp, hatched; pairing segment, black; A, autosome; $\blacktriangle$ inactivation centre; $\bullet$ testis-determining gene. Arrow shows the extent of spread of X inactivation. The genetic content of the homologous region of the platypus X is not yet known. The proposal in this article is that Ohno's law operates on those genes covered by the spread of inactivation.

may rise in future. Thus, among mouse chromosomes the X is indeed exceptional in having homology with only a single human chromosome, the X. Watson *et al.* suggest that rodents may be unusual in the number of chromosome rearrangements in their evolution, and certainly among the human autosomes there are two which have known homologies on only a single mouse chromosome, human chromosome 17 on mouse 11 and human 20 on mouse 2 (ref. 7). But the situation in the mouse, with the X being the only chromosome conserved intact, seems to provide support for Ohno's Law.

How then can the positions of X-linked genes on the platypus X be explained? Although the mechanism of X inactivation is not understood it is generally believed that the process begins from an inactivation centre and spreads along the chromosome^{8,9}. Graves and her colleagues^{2,4,6} have suggested that in the course of evolution the length of the inactivated region, and hence the distance of spread, has increased gradually as the X and Y became differentiated, with the reduction of the Y. The monotremes are considered as showing a primitive state. So it is possible that the present order of genes in the platypus X is a primitive one and that the group of genes including G6PD has been recruited into the differential segment in the line of descent leading to the placental mammals and marsupials. It would be valuable to know whether the three genes have homologues on the Y, but Watson *et al.* were not able to obtain this information.

On the other hand, it might be that a relatively recent rearrangement has moved G6PD from the differential segment to the homologous region. There is evidence of rearrangement in the monotremes as a group as the other two species have an X_1X_2Y system of sex determina-

tion, in contrast to the platypus XY system⁶. If the G6PD group had been so repositioned what then would be the state of activity in these genes? Unfortunately, it is not yet possible to study X inactivation in monotremes in detail. There is no sign of asynchronous replication of these genes, but the segment might be too small to see. If the genes were indeed inactivated it would mean that the inactivation signal had spread from the putative inactivation centre through a large non-inactivated homologous segment. There is precedence in the human for inactivation of genes beyond a non-inactivated region^{10,11}.

Watson *et al.* suggest however that spreading would extend over shorter distances in monotremes and this seems a reasonable hypothesis. Thus, the genes might remain non-inactivated, and would then be behaving like genes in mice physically separated from the inactivation centre by an X-autosome translocation^{8,9}. Such genes remain active. This in turn might provide a new insight into the operation of Ohno's law in the early stages of the evolution of X inactivation. There may have been a stage when X inactivation covered only a small part of the X, as in present monotremes (see figure). Rearrangements could then occur which

transferred inactivated genes beyond the reach of the spreading signal. They would spontaneously lose their inactivation, and if by crossing-over they obtained a homologue on the Y they would have no dosage problems. Translocation to an autosome would then be possible.

It is tantalizing that genetic investigation of monotremes is so difficult. Further knowledge of the inactivation or not of G6PD, and a possible homologue on the Y, in the platypus could provide interesting further insight into X inactivation and its evolution. $\square$

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COMPUTATIONAL PHYSICS

Of micelles and membranes

Stanislas Leibler

FROM the point of view of equilibrium thermodynamics, a binary mixture of two simple liquids can form either a homogeneous phase or a heterogeneous state in which the two fluids are separated by a macroscopic interface, as happens in mixtures of oil and water. But if the molecules of one or both liquids are more complex, all sorts of thermodynamically distinct phases can arise. For instance, a binary mixture of amphiphilic molecules (bearing both hydrophilic and hydrophobic parts) and water can form many different molecular assemblies such as micellar solutions, suspensions of bilayer vesicles, stacks of bilayer membranes, liquid crystals with cubic or tetragonal symmetries¹. This rich polymorphism has generated widespread interest in trying to explain its origin from some microscopic description of molecules and their mutual interactions. An important class of theoretical approaches to the behaviour of molecular assemblies involves numerical simulations.

The paper by B. Smit and colleagues on page 624 of this issue² exemplifies such an approach. The authors use a modern tool of numerical computing, namely a parallel network of 100 transputers, to undertake a molecular-dynamics study of a three-

component liquid mixture. The form of interactions between the molecules is chosen to mimic the behaviour of oil-water-surfactant mixtures. The authors simulate some of the simplest structures observed experimentally in such mixtures: surfactant monolayers at oil/water interfaces and surfactant micelles (miniature globules) inside oil regions. Moreover, these structures coexist with one another in a single simulation, in accord with the experimental evidence.

More interestingly the coexisting micelles and films effectively repel one another although no explicit term representing molecular repulsion between the hydrophilic heads of the surfactants has been introduced into the model. The effective force seems to be analogous to the entropic repulsion predicted to exist³ between fluctuating membranes. Two extended thin membranes brought close together mutually hinder each other's undulations, restricting the possible configurations between them. Because the effective repulsion that results is of entropic origin, molecular details should not alter it. For example, the repulsion varies as A/l^2 where l is the separation and the prefactor A depends only on the temperature and the membranes' macro-

scopic elastic properties³. Indeed, this 'universal' law has been verified in X-ray diffraction experiments⁴. Although it would be interesting to compare simulations of such forces for micellar systems with experimental data, such a quantitative comparison could be hard. In the case of micelles, which are rather small objects, the entropic forces surely include non-universal factors which could depend on the structure of water, oil or surfactant molecules, a structure which is far from being realistically taken into account.

This brings us to a much larger question: how far can we go in numerical simulations of complex molecular structures and what can we learn from them? Despite considerable increases in computing power over recent years, realistic simulations are possible only for the simplest aggregates⁵. The timescales involved in even the largest molecular dynamics simulations are much too short to describe the formation and thermodynamic behaviour of large assemblies of molecules.

One way to overcome this impasse is to go even a step further in simplifying the models and concentrate the attention on more macroscopic and universal properties of the aggregates. In such an approach, F. F. Abraham and D. R. Nelson^{6,7} have recently reconsidered a model of a single fluctuating membrane in which, instead of starting with molecules and their mutual interactions, they simulated an already assembled membrane, described in terms of an arbitrary two-dimensional lattice by an effective phenomenological set of interactions. An important parameter which characterizes this fluctuating sheet is its bending modulus, κ , an elastic constant which for real membranes depends on the detailed molecular structure. Its value, or rather its ratio $\kappa/k_B T$ to the thermal energy, largely determines the shape, fluctuations and other properties of the membrane, although other factors — the nature of the internal connections in the sheet for example — also play a role. For instance, the 'tethered sheets' simulated by Abraham and Nelson, which are analogues of polymerized membranes, behave differently from fluid membranes. They are found in a so-called flat phase, characterized by a long-range orienta-

tional order and very unusual elastic properties⁹. For instance, the spectrum of undulations and phonons (vibrations) and the response to external forces are strongly modified compared with the usual, 'classical' laws. The analytical predictions seem to be in agreement with the numerical work. The ability to apply analytical tests¹⁰ to the simulations is important in confirming that they represent equilibrium states, as well as in extrapolating their results from small to larger systems.

Such molecular-dynamic or Monte Carlo simulations could in fact help to understand the thermodynamic behaviour of many large molecular aggregates (different membrane structures, gels, cytoskeletons and so on). Although these simulations do not include molecules with

any realistic forms or interactions, they do shed the light on some collective properties of the aggregates, such as their behaviour at length scales larger than the molecular sizes. If one is interested, for instance, in simulating the shapes, fluctuations, fusion events or mutual repulsion of large vesicles¹¹ it is impractical to start with an ensemble of realistic molecules. From this point of view, Smit and coworkers' simulations, and other similar studies of micelles and membranes, could constitute a bridge between the truly microscopic and the phenomenological, macroscopic approaches. □

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DEVELOPMENTAL BIOLOGY

Fly fishing downstream

Gines Morata and Gary Struhl

THE homeotic genes were the first developmental genes recognized in *Drosophila*. That a single mutation can cause one appendage to develop as another, such as the transformation of antenna to leg caused by mutations in *Antennapedia* (*Antp*), is immediate evidence that the gene concerned has an important regulatory role. Indeed, the products of these genes are sufficient in themselves to activate genetic programmes controlling the development of cell patterns as complex as that of the wing, leg, eye or antenna. Moreover, only seven or eight homeotic genes like *Antp* (clustered in the *Antennapedia* and *Bithorax* complexes) manage to control the distinct developmental pathways followed by at least 15 metameric primordia giving rise to the segmented portions of the larva and adult (reviewed in ref. 1). We know that the initial realms of action of the homeotic genes are subsets of these primary embryonic metameres (parasegments 1–15; ref. 2) and that the characteristic pattern of each parasegment depends on the combination of active homeotic genes³ and on the cell-by-cell deployment of their protein products⁴. Gould and colleagues, whose report appeared in *Nature* last month⁵, now take us a step further forward with the pioneering of a novel and apparently successful approach for identifying putative target genes of homeoproteins in *Drosophila*.

Homeotic genes must control cell pattern by governing the spatial patterns of expression of downstream 'realizator' genes⁶. Indeed, the homeotic genes of the *Antennapedia* and *Bithorax* complexes encode nuclear proteins with a DNA-binding motif, the homeodomain, implying that they directly regulate the transcription of the downstream genes. But we still cannot explain how homeotic gene

action is translated into the development of particular cell patterns. For example, we don't know whether homeotic gene products directly regulate the localized activity of all of the downstream genes involved in the arrangement and development of individual components of the pattern (bristles, hairs, veins and so on), or, alternatively, whether they sit at the top of a hierarchical cascade, several steps removed from the final realizator genes (as suggested by the recent finding that *Ultrabithorax* (*Ubx*) influences visceral mesoderm development by regulating the putative signalling proteins *wingless* (*wg*) and *decapentaplegic* (*dpp*)⁷). Similarly, we cannot explain how presumed target genes that are likely to be active in all parasegments (for example those involved in specifying where sensory neurons differentiate) are expressed in different patterns depending on which homeotic gene products are present.

The key to understanding how homeoproteins create morphological diversity lies in determining how they control the spatial expression of downstream genes. Yet little progress has been made in identifying these genes or understanding their regulation. Genetic methods have not been very successful, for it is not clear what phenotypes to screen for. Mutations in such genes are also likely to be lethal, further complicating genetic approaches to identifying them. A few candidate target genes may exist among previously identified developmental genes (for example *wg* and *dpp*), but further analysis is necessary to establish whether they are direct targets. Similarly, the enhancer trap method⁸ offers the possibility of seeking for transcriptional enhancers which respond differentially to homeotic gene activity, although it is unclear how many

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candidate genes identified by this approach will be direct targets.

Gould *et al.*⁵ describe a new, more direct, molecular approach based on the knowledge that homeotic genes encode transcriptional regulators which bind DNA directly via the homeobox domain. Unfortunately, identifying target sites by the ability of *Ubx* protein to bind them *in vitro* is compromised by the potential lack of specificity of DNA-homeoprotein interactions observed under these conditions. To obviate these problems, Gould and colleagues make use of a highly specific monoclonal antibody that recognizes all *Ubx* protein products⁶. They prepare embryonic chromatin at low ionic strength to preserve the native structure so that the *Ubx* protein will be able to bind or remain bound to its *in vivo* DNA targets, and then immunopurify the soluble fraction with anti-*Ubx* antibody. DNA from the immunopurified fraction is then digested and cloned in small fragments.

It turns out that this population of clones is enriched in sequences matching the consensus TCAATTAAAT to which many homeodomain proteins bind *in vitro* with high affinity^{10,11}. Moreover two of four clones chosen for further analysis, numbers 35 and 48, contain sequences to which *Ubx* protein binds *in vitro* with high selectivity. To test whether these clones are actually target sites of downstream gene expression, the authors fished out the associated transcription units and assayed where they are expressed in wild-type and mutant embryos. Strikingly, clones 35 and 48 are both associated with transcription units which are expressed in a segmentally repeated pattern. More importantly, both transcripts show distinct patterns of expression in different parasegments and the differences are dependent on *Ubx* gene function. These results strongly suggest that the method has worked and that two *Ubx* target genes have been positively identified.

An additional and important observation that Gould *et al.* have made is that the two genes also seem to be regulated by other homeotic genes. This is certain for clone 35, which appears to be independently down-regulated by the other genes of the bithorax complex (*abdA* and *AbdB*), and is likely to be the case for clone 48, which also seems to be regulated by homeotic genes in the Antennapedia complex. The possibility that some downstream genes are independently regulated by several homeotic genes makes a lot of sense. It is consistent with the strong amino-acid similarity found in the DNA-binding motifs of homeoproteins, and also provides an explanation for the observation that some homeoproteins can suppress the phenotypic consequences of the activities of other such proteins. For example, the fact that the *Ubx* protein can dictate a parasegment-6-specific pattern

in the presence of *Antp* protein¹² is better understood if *Ubx* and *Antp* proteins compete with differential affinity for binding to largely overlapping sets of downstream genes.

The obvious course of action from here is to determine the developmental and molecular function of the two candidate genes already identified. As they have been cytologically mapped to the 64C and 97D regions of the chromosome, it should be possible to alter them by conventional genetic techniques and assess what happens when their function is eliminated. Similarly, it should be relatively easy to determine their sequence, which may reveal aspects of their molecular structure and function. Another direction will be to identify more candidate genes. In principle, the method devised by Gould *et al.* could be used to find out whether pre-existing candidate genes such as *wg* and *dpp* are direct targets *in vivo*. Perhaps more importantly, the method could allow a comprehensive assessment of the numbers and types of target genes which are directly regulated by a given homeotic gene. However, one point to bear in mind is that one of the four genes initially selected by these authors (gene 9) is not likely to be a target of *Ubx* *in vivo* as it is expressed at high levels in parasegment 15 where the *Ubx* protein is not normally present. So it is possible that *Ubx* protein can dissociate and reassociate with target sites in chromatin under the conditions of the experiment, opening the possibility for artefactual associations between the protein and inappropriate target sites.

But the approach developed by Gould *et al.* could lead to significant advances in understanding how homeoproteins control cell pattern. It should also be applicable to other classes of regulatory proteins and to other developmental systems — a lure, perhaps, for other anglers. □

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Micromagnetics

AMBITIOUS chemists are already dreaming of 'molecular electronics', whose components are single molecules. Daedalus recalls that one molecular superconductor is already well known: the ring molecule of benzene. Studied by nuclear magnetic resonance, its six-carbon ring proves to have a current flowing endlessly around it, which varies with the applied magnetic field. Daedalus points out the obvious reason: the molecule acts as a tiny one-turn superconducting coil. Pushing it into the magnetic field induces a current which suffices to exclude the field from the ring.

What would happen, asks Daedalus, if benzene was synthesized in a magnetic field? Each newly formed superconducting ring molecule would have a few flux lines of the field threaded through it. There would be no ring current, but the superconducting ring molecules could never cut through the flux lines to escape. They would be permanently threaded on the lines of force like loose beads on a string.

The consequences would be fascinating. For a start, the benzene could not escape from the synthesis magnet even by evaporation. Each molecule could only move along the line of force which was threaded through it. The molecules would jostle endlessly back and forth between the magnetic poles like a sort of one-dimensional gas, and would be permanently trapped. They might even exert a considerable end pressure on the poles.

So Daedalus proposes a novel 'benzene cushion' hovercraft, whose downwardly directed magnetic field intersects the ground beneath it. *In situ* synthesized benzene vapour, trapped in the field and exerting its saturated vapour pressure of about 0.1 atmospheres, would float the craft above the ground, silently, continuously and without expenditure of energy. This wonderful vehicle, the ultimate in frictionless movement and suspension, would revolutionize transport — provided that smoking was strictly prohibited. A flame would burn the benzene to non-magnetic carbon dioxide, which would immediately escape around the edges of the craft and let it down with a bump.

Even more intriguing, suppose benzene was synthesized between the poles of an electromagnet, which was then switched off? The contracting lines of force could no longer close on themselves and vanish; they would have thousands of benzene molecules packed along them like beads on a circular elastic necklace. The result would be a novel 'poly-benzene' held together, not by chemical bonds, but by lines of magnetic force. It would be a sort of 'portable magnetic field'. Brought near a piece of iron, its flux lines would penetrate it to recreate a magnet with benzene-loaded lines of force again.

David Jones

Enzyme motifs in antibodies

SIR—The promise of protein crystallography for understanding the structural basis for enzyme mechanisms is being realized, in part, by the growing recognition of a few basic catalytic motifs. Examples include the structurally related Asp-His-Ser and Asp-His-Zn catalytic triads, recently discussed in *News and Views*¹ and in *Scientific Correspondence*².

Conventional studies of enzyme active sites use site-directed mutagenesis to probe the chemical function of amino-acid residues. We have developed a complementary approach involving transplantation of catalytic motifs into antibody-combining sites which should clarify the function of residues in enzyme catalytic motifs and improve the design of catalytic antibodies by allowing the addition of appropriate residues and metal cofactors.

Although other proteins with catalytic sites have been designed *de novo*, such as a helix bundle with serine protease activity³, the use of antibodies has several advantages. First, the structure of the antibody framework is strongly conserved, providing a well-defined protein backbone. Second, portions of the complementarity-determining regions are strongly structurally conserved but allow for sequence variability⁴, so catalytic models can be built in a predictable manner. Third, the



Transplantation of a cofactor-binding site into the antibody light-chain complementarity-determining regions (CDRs). The framework Fv regions (dark grey) for the antibodies McPC603, D1.3, HyHEL5, HE10, Newm, Kol, J539, 4-4-20 and R19.9 are superimposed to show the C₁ backbones of the light-chain variable region (CDR1, medium grey; CDR2, white; and CDR3, light grey) with the zinc site from carbonic anhydrase (three histidines and the zinc atom) superimposed onto residues 34, 89 and 91. Binding site (top) faces forward. Although the CDRs show much more structural variation than the framework regions, all the antibodies share the β -strand structure that extends from the framework into the CDRs.

antibody sequence can be varied in these structurally conserved regions to test the specific requirements of the environment surrounding the catalytic motif. Fourth, the immune system can be used to create antibodies with appropriate binding sites for a given antigen. Finally, as techniques are now available for the combination of separate light- and heavy-chain libraries, chains with different binding specificities can be matched to complementary chains with different catalytic motifs.

Structural templates can be recognized, dissected from their environment in the enzyme and transplanted into any of a set of general sites within the antibody-combining site⁴ where their resulting metal binding or catalytic activity can be tested outside the original enzyme. Because these general sites are in the structurally conserved, sequence variable areas of complementarity-determining regions, this transplantation process is likely to be applicable to all antibodies.

An example of a structural template is the three-dimensional pattern of catalytic zinc sites, which include a main-chain hydrogen bond joining two of the zinc ligands⁴. The template for carbonic anhydrase, in which the three zinc ligands lie on two hydrogen-bonded antiparallel β -strands, can be accommodated in five structurally conserved sites general to all known antibody structures⁴. For one such antibody light-chain general site, the replacement of three amino acids in the complementarity-determining regions with histidine residues formed a zinc-binding site with an open coordination position at the bottom of the antigen-binding pocket (see figure). Creation of this site in the anti-fluorescein antibody 4-4-20 gave a mutant antibody that binds copper, zinc and cadmium with the same preferences as carbonic anhydrase⁵.

Thus, multisite design based on structural templates can be used to remodel an antibody to contain a cofactor-binding site, without requiring further mutagenesis and screening. Our new approach for designed antibody mutants could be used to evaluate features such as the Asp-His linkage in Asp-His-Zn and Asp-His-Ser catalytic triads independent of other features in the evolved enzyme active site.

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Alcohol sensitivity

SIR—Luddens *et al.*, in their paper¹ on the importance of the $\alpha 6$ subunit in the binding of the benzodiazepine Ro15–4513 to a subtype of the GABA_A receptor, stated: “most importantly, the newly characterized GABA_A receptor seems to be functionally involved in mediating the alcohol-induced impairment in motor performance that can be antagonized at the same receptor by Ro15–4513”. Because the authors found this subunit only in cerebellar granule cells, their conclusion is not consistent with the fact that the authors of the original study showing antagonism of ethanol on GABA_A receptors by Ro15–4513 (ref. 18 in ref. 1) used rat cortex, a tissue that does not contain the $\alpha 6$ subunit. Furthermore, we have shown² that Ro15–4513 is equally effective as an ethanol antagonist with GABA_A receptors from cortex or cerebellum.

In the cerebellum, ethanol-induced motor impairment is genetically linked with the action of ethanol on Purkinje cells (which do not contain the new receptor)^{3–5}. This action is antagonized by Ro15–4513 (ref. 6). It is possible that the $\alpha 6$ subunit is expressed in other brain regions and cell types and that the techniques used by Luddens *et al.* were not sufficiently sensitive to detect this expression, or that it is developmentally regulated (the authors did not specify the age of the rats used) — but these hypotheses remain untested.

Finally, although the Ro15–4513 binding site does differ in one pair of lines (AT/ANT) selected for ethanol sensitivity (ref. 17 in ref. 1), no differences were detected in this site in eight other lines (LS/SS, HAS/LAS, FAST/SLOW, HOT/COLD) selected for alcohol sensitivity (E. R. Korpi, personal communication), although differences in ethanol sensitivity of the GABA_A receptor have been shown for several of these lines^{7,8}. Thus, the difference in receptors between AT/ANT rats may result from gene fixation during inbreeding and may be unrelated to alcohol sensitivity. In view of these data, it seems premature to attempt to link the $\alpha 6$ subunit to alcohol sensitivity.

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Random fingers

SIR—Edwards (*Nature* **246**, 419; 1990) points out the subjective element of describing a pattern, which raises an interesting practical problem in the use by police forces and others of fingerprints as a method of identification. Modern technology now offers the possibility of automatically searching large collections of fingerprints, with considerable benefits in time saved in making identifications.

The economical storage and transmission of fingerprint images requires electronic processing, during which some of the information contained in the original image is necessarily lost. At some point, depending on the extent of this processing, so much information is lost that the processed image can no longer be said to resemble the original. The point at which this occurs is perceived differently by expert fingerprint officers and non-experts; the former are far more discerning and can identify very small differences.

The nature of a fingerprint image makes it especially sensitive to information loss from key areas, which makes simple comparisons of correctly and incorrectly recorded pixels useless as measures of information loss. An objective way of measuring the information content and hence information loss from fingerprint images would be of considerable value in the design and evaluation of automatic fingerprint identification systems.

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Polarized views

SIR—Derek Fordham in his review of the Navigation Foundation's report *Robert E. Peary at the North Pole* (*Nature* **344**, 902; 1990) asserts that it contains no new information, only new analysis. We did indeed report little new information but identified a need for competent analysis. For example, we confirmed Will Steger's duplication of Peary's mileages, discredited by Wally Herbert, by correlating satellite location data with daily log entries, verified by Steger. Our use of Peary's depth soundings to show by current bathymetric data that he was on track to the Pole, was a first.

Also unique was our application of photogrammetry to Peary's photographs. When Fordham says our shadow analysis suffers from uncertainties of time of day at which the pictures were taken, he overlooks our determination of approximate times derived from analysis of the pictures together with Peary's captions. There is a unique characteristic of the Sun's diurnal

path at the poles independent of date or time. At latitudes a degree or more less than 90 degrees the path is inclined so that solar altitudes measured approximately 16 hours apart, as in this case, differ by an order of a degree or more, because the difference will depend on the latitude and the change in declination. By contrast, the difference at the Pole is quite small, being only the changed declination, about one quarter of a degree.

We have now published a supplement to our report which contains two pictures, each showing both the Sun and the horizon, thus permitting direct measurement of the Sun's altitude. These altitudes agreed to within one-tenth of a degree with those from the shadow analysis, enabling us to show the altitude change was only that expected due to changing declination, and therefore that the pictures had been taken at or very near the pole.

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FORDHAM REPLIES—In view of all the evidence available, some of it in the Navigation Foundation's own report, and the points I made in my review, I remain unconvinced by the new information. Further, the mileages achieved on Will Steger's expedition do not confirm Peary's. On the last stages to the pole, from the latitude where, on Peary's expedition, Bartlett turned back, Steger achieved a daily average of 18.8 nautical miles per day latitude distance whereas Peary claimed 26.

The bathymetric analysis relies on a small-scale computer-generated ocean-floor map on which it is impossible to be as precise as the Navigation Foundation would wish. It can be interpreted to support either case. The foundation's report confirms uncertainties as to the time when Peary's polar photographs were taken. Accurate time is the essence of position-finding navigation and cannot be derived from examining photographs whose exact bearings are unknown.

At the pole, the Sun appears above the horizon in mid-March and rises in an ascending spiral to an altitude of approximately 23° 30' on about 22 June. It is only on that date, by which time Peary had been back on board his ship for 2 months, that the Sun followed the almost level course Davies describes above the Pole. In no way is the Sun's path, at the pole or anywhere else, "independent of date or time".

In fact, on 6 April 1909, when Peary claimed to be at the pole, the vertical component of the Sun's angle of ascent was greater than average and amounted to approximately 23' per day. This is why it is necessary to know the exact time before attempting to use such photographs for

position fixing. In the 18-hour period during which the foundation assesses the polar photographs to have been taken, the altitude of the Sun would have changed by a minimum of 17' which, if ignored, would give an error of up to 17 nautical miles in any position line based on an assumption of constant altitude.

I believe that the application of a very precise photogrammetric technique to photographs for which no similar precision in time or bearing is available cannot produce a result of sufficient accuracy to confirm Peary's presence at the pole. One degree out in the calculation of the Sun's altitude from lines drawn through estimated horizons or from objects to shadows could vary the position line by 60 nautical miles. Together with all the other uncertainties which still surround Peary's claim, such a variation could have placed him just about where Herbert claims he was on 6 April 1909.

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Long tree-rings

SIR—The 1,500-year tree-ring reconstruction of Fennoscandian temperature is actually not the "longest annually resolved climate reconstruction from tree-rings yet published", as claimed in ref. 1, but is surpassed by the 1,614-year tree-ring reconstruction of the Palmer drought index for North Carolina². These two reconstructions can claim bragging rights as the longest annual tree-ring reconstructions so far produced, but much longer, absolutely dated tree-ring chronologies exist and are sure to provide millennia-long palaeoclimate reconstructions in the near future.

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Scientific Correspondence

Scientific Correspondence is intended to provide a forum in which readers may raise points of a scientific character. They need not arise out of anything published in *Nature*. In any case, priority will be given to letters of fewer than 500 words and five references. □

All in his head

Robert Temple

Essays on Science: Felicitation Volume in Honour of Dr. Joseph Needham. Edited by Hakim Mohammed Said. *Hamdard Foundation Pakistan, Karachi, Pakistan: 1990.* £20, \$40, Rs 400.

A Selection from the Writings of Joseph Needham. Edited by Mansel Davies. *The Book Guild Ltd., (Lewes, Sussex, England).* Hbk £25.

JOSEPH Needham is probably the world's greatest living scholar, and on the occasion of his ninetieth birthday in December it is fitting that these volumes should appear. The interesting volume of *Essays* from Pakistan is well worth acquiring, if one can bear the maddening refusal by the Indian and Pakistani contributors to use the definite article or to have correct English grammar. Only one contribution is in French, and that a brief one; all else is in English, or some semblance of English. Needham's collaborators, Kenneth Robinson, Dieter Kuhn and Gregory Blue, contribute respectively an advance section of *Science and Civilization in China* on literary Chinese as a language for science, on a side-avenue of the history of textiles, and a foretaste of H. T. Huang's study of the history of noodles and spaghetti. Important articles are included by Debiprasad Chattopadhyaya on the early Indian empirical scientist Uddālaka, who predated the Greek Thales and has a stronger claim to being the 'earliest scientist' outside of China; by Pan Jixing on the invention of the rocket, in which he adduces additional support for Needham's position; by Georges Métaillé on the botanical terminology of Li Shihchen, China's greatest natural scientist (seventeenth-century); an extraordinary account of the Harappan unicorn by B. V. Subbarayappa; a survey of Indian science by S. N. Sen; a surprising study of the origins of Japanese managerial reform by Tetsuro Nakaoka; and an excellent survey and appreciation of Needham's work by the editor. The book is a fascinating collection of diverse subjects in the history of science of India, China and the Arabs, much of it relating to Needham's work. It should be on the shelf of all students of these subjects.

The selection of excerpts from Needham's voluminous writings represents his work other than the gigantic *Science and Civilization in China* series, from which excerpts have not been taken. Not every book of his, much less every article, is

actually represented. For instance, his *Man a Machine* of 1927, which was his first monograph, is not even mentioned; the book has no bibliography of Needham's writings other than to list the fifteen works consulted for extracts. The editor did persuade Needham to allow one of his sermons to be published for the first time,



Needham — a genius almost without parallel in our time.

this one delivered at Caius College in 1976. Over many decades Needham has been a lay preacher at an Anglican church at Thaxted in Essex, and there are mountains of his sermons in existence. I once asked him if he ever intended to have them published, and he said "Only after I am dead, if anyone wants to." This is therefore the first appearance in print of this side of Needham's multivarious persona. Extraordinarily useful also is Needham's autobiographical essay "The Making of an Honorary Taoist", which has appeared on previous occasions but is most welcome here, as it is a humorous attempt at self-analysis by a kind of alter-ego of Needham's christened Henry Hollerenshaw, a fictitious Derbyshire name which he first used to write a pseudonymous book entitled *The Levelers and the English Revolution* (1939). Needham told me once he did not dare use

his own name on the book because he imagined it might endanger his becoming a fellow of the Royal Society, as "the Royal Society do not like people having too broad interests and might take against me for it so that I might not get in." Long extracts from this book are given, although Needham is not infallible on this subject, failing to draw sufficient distinction between the tiny Digger movement and the larger Leveller movement, and wrongly imagining that the latter were precursors of socialism, which was only true of the former. (But there are many who agree with him, and I once had an earnest lecture on this subject from Tony Benn, which could have come straight from the pages of Needham, and possibly did.)

The weak point in Needham's writings is certainly political. In *The Grand Titration* he speaks of "the inevitable transition from capitalism to some such economy as that of the Soviet Union, where . . . each man's . . . participation in the government of the . . . state is acknowledged." This was silly at the time (1944, during the oppressive regime of Stalin), but today it is a monstrous aberration in an otherwise great intellect. Needham has told me he was "never a communist" and defines himself today as an "international socialist". There certainly are not very many of those left east of the River Neisse today! For a man otherwise a genius almost without parallel in our time, Needham has been curiously misguided in his political philosophy.

The magnitude of Needham's intellectual achievements is so gargantuan that, having read 8.5 million words of his at least, I still find that he has surprises to spring. It is as well to remember that he is fluent in at least eight languages, three of them ancient, and once gave a speech in Polish. Perhaps the best anecdote is what his first wife Dorothy said to my friend Peter Mitchell many years ago in Cambridge. Referring to Needham's photographic memory, and the proofs of an early volume of *Science and Civilization in China*, 'Doffy' said: "Joseph has a new game. He used to lie in bed correcting his proofs in his head. But he got bored with that. So now he translates them into French first." (In his head, of course.) And it is this head of Joseph Needham that gives us hope for the future of the human brain. □

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On the beam

Alan Phelps

Undulators and Free Electron Lasers. By P. Luchini and H. Motz. *Oxford University Press: 1990. Pp.322. £40.*

HANS Motz invented the undulator in 1951 and was therefore uniquely well qualified to write a book on the subject. Although Motz died in 1987 this book represents his collaborative writing with Paolo Luchini in the immediately preceding years. Luchini completed the book after Motz's death.

An undulator is a device that presents a spatially periodic magnetic field to an

Doppler upshift and intentionally operated in the microwave region.

Luchini and Motz have concentrated on the short wavelength FEL in contrast to the microwave FEL which has been given more space in the book *Free Electron Lasers* by T. C. Marshall. Marshall's book is aimed at a less mathematical readership than the book of Luchini and Motz, which retains mathematical rigour when the results justify it. The authors have successfully written a book suitable as a learning tool for scientists or graduate students wishing to enter the field and also suitable as a reference text for those already actively working on FELs. To cater for those readers not familiar with some of the elements of hamiltonian relativistic mechanics and classical electromagnetic

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Star Wars research — high-power laser beams form part of the US Strategic Defense Initiative.

electron beam and thereby generates a beam of electromagnetic radiation. Effectively it concentrates the electron synchrotron radiation into a narrow cone and into a narrow frequency interval. Although the radiation is incoherent it can be made coherent by placing the undulator in a cavity and providing feedback of the electromagnetic radiation field to bunch the electrons into short packets with linear size comparable with the electromagnetic radiation wavelength. The term 'free electron laser' (FEL) was introduced by John M. J. Madey in 1971 and the first self-oscillation of an FEL was achieved in 1977 by Deacon, Elias, Madey, Schwettman and Smith at Stanford. Since that date several FELs have operated successfully. The use of highly relativistic electron beams allows the emitted electromagnetic radiation to be Doppler upshifted from the centimetric dimensions of the undulator to the visible spectral region. The first chapter provides a general overview of the subject through a description of the arguments and results obtained in some of the early papers. This chapter also includes a brief discussion of the Ubitron which was built in several versions by R. M. Phillips in the period 1958–65. This device did not have a large

radiation theory, a brief introduction to both has been provided.

After treating the motion of an electron in an undulating field and considering the properties of undulator radiation, this book devotes chapters to the small-gain FEL, large-gain FEL and saturation. Luchini and Motz then discuss tapered undulators, which have become increasingly important for operation of FELs at maximum efficiency. The authors also point out that the undulator does not only have the FEL as an application, but also allows the operation of the inverse FEL. The latter device may also be regarded as a laser-driven particle accelerator. The last chapters are usefully directed towards the practical subjects of undulator operation, magnet design and experiments and applications.

One has the feeling, when reading this book, that it is destined to become a physics textbook classic. It is likely that all libraries in institutions that specialize in laser research and more specifically free electron laser research will want to acquire a copy of this important text. □

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Variety club

C. D. Pigott

Ecology of Plant Communities. By J. O. Rieley and S. E. Page. *Longman: 1990. Pp.178. £25.*

MOST British plant ecologists have resisted the production of a comprehensive classification of British vegetation by the methods of plant sociology. They have claimed either that the methods are subjective and theoretically flawed, or that classification freezes an essentially dynamic system and diverts attention from the real understanding of the ecology of plants.

But for practical purposes the lack of a comprehensive and generally accepted classification of British vegetation has been a serious obstacle both to the development of experimental ecology and to the application of ecology to a wide variety of problems. Ecology cannot, by definition, be the study of plants in isolation, but must include the study of their responses within the vegetation of which they are a part. This has clear practical application in nature conservation, where it has become increasingly apparent that it is the variety of types of vegetation which has to be conserved. Then, to a large extent, the variety of species of both plants and animals, including those which are rare, will look after themselves.

It might be supposed, therefore, that the appearance of a textbook, which provides a treatment of the methods of plant sociology and a phytosociological account of British vegetation, would be more than welcome: particularly a book which is so well presented and attractively illustrated. Unfortunately, it seems to have been written by authors who are either unaware of events in recent years, or who have chosen to ignore them.



Plant classification — an obstacle to a real understanding of plant ecology.

In 1975 a contract was awarded by the Nature Conservancy Council to the University of Lancaster to prepare a comprehensive account of British vegetation. With the cooperation of many other workers this National Vegetation Classification has been conducted with considerable publicity and in January 1991 the first volume will be published, to be followed in rapid succession by the remaining four volumes. No reference to this venture appears in the book and, as a consequence, the classification which the authors have set up simply by equating British vegetation to that described on the Continent will differ substantially from the system about to be published. The latter has been largely based on analyses of existing vegetation and classification using multivariate techniques. Not surprisingly, some British types of vegetation differ considerably from their continental counterparts and it may not be appropriate to refer to them by the same name.

Even if the authors are forgiven for supposing that the gestation period of the National Vegetation Classification has

been so long that a stillborn result could now be expected, their omission of any reference to computerized methods of handling data is remarkable. The techniques described in chapter 3 could have been written half a century ago and indeed convey much the same information as in the English translation of Braun-Blanquet's *Plant Sociology: The Study of Plant Communities* (McGraw-Hill; 1932).

It seems that an opportunity has been missed to provide a much needed introductory textbook which would have prepared students to accept the methods and classification of the National Vegetation Classification which are already widely in use. There is the risk that it is counter-productive because it sets up two different systems of nomenclature and will make students justifiably irritated, in much the same way as do the apparently unending changes of the scientific names of plants. □

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Poison gas

Matthew Meselson

The Challenge of Chemical Weapons: An American Perspective. By Victor A. Utgoff. Macmillan: 1990. Pp.273. £35.

THE most valuable contribution of this book to the literature on chemical weapons is suggested by the subtitle, 'An American Perspective'. As a former staff member of the US National Security Council and chairman of the Interagency Group on Chemical Weapons in the Carter Administration, Victor Utgoff was well positioned to note the views of the relevant US government agencies regarding chemical weapons and chemical disarmament. After a brief and generally useful account of the history of chemical weapons and chemical arms control efforts, the book focuses on US policy for chemical weapons — a policy that has two rather different components. One is the effort to produce and maintain chemical weapons as a like-for-like deterrent and retaliatory force. The other is a pursuit of a global chemical disarmament treaty as a means to prevent proliferation and to eliminate or substantially reduce the already existing chemical threat. This duality leads Utgoff to define a major challenge he sees facing US policy. How, he asks, can support be maintained for a US retaliatory chemical capability sufficient to deter the Soviet Union until an effective chemical arms ban enters into force?

Framed this way, the question appears moot. Since Utgoff wrote, the Warsaw

Treaty Alliance has disappeared as a military entity, US chemical weapons have been withdrawn from Europe and the US binary chemical weapons production programme has been cancelled. In view of these changes, one might think this most distinctive part of the book would be of historical interest only. Not so. US policy continues to be dominated by the tension between chemical deterrence and chemical disarmament. Some in Washington argue that the Soviet threat has been replaced with present and possible future chemical threats from the Third World. This approach underlies Defense Department support for the current US proposal

at the chemical disarmament negotiations in Geneva that would allow it to retain two per cent or about 500 agent tons of its chemical weapons stocks indefinitely, until it is satisfied that all other "chemical weapons capable" states have joined the treaty. Because the idea of such residual stocks is strongly opposed by many of the nations negotiating the treaty, insistence on it could defeat the entire enterprise. Whether or not one finds the military case for retention of a US chemical deterrent convincing in this newer context, Utgoff's presentation remains of interest.

Utgoff makes the familiar argument that although antichemical protective measures can keep chemical casualties to militarily negligible levels, the burden of wearing chemical protective gear and taking other necessary precautions will seriously interfere with the performance of specific types of combat activity. The more novel part of the presentation is a consideration of the effects of chemicals on thirteen different target types in a NATO-Warsaw Pact confrontation in Europe. In that hypothetical context, chemicals are seen as of low effectiveness against mechanized targets and targets with collective antigas protection, such as tanks, self-propelled artillery, rocket and missile launchers and field headquarters, but of high effectiveness against air bases, military storage and repair sites and infantry engaged in dismounted assault. Chemical weapons are accordingly portrayed as necessary for deterrence of chemical attack and for possible retaliation, to force an adversary into a level of protective posture similar in its effects to that which his chemicals impose on ones' own forces.

Missing from Utgoff's analysis is any evaluation of the arguments against a policy of chemical deterrence. What

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Chemical warfare — tension still exists between deterrence and disarmament.

about those field exercises that, contrary to what is usually said, have shown little effect of antichemical protective posture on overall military performance? How significantly might the impact of chemical weapons be further reduced by improvements in defensive equipment, exemplified by the newer European NATO and Israeli suits and masks? And how does one assess the importance in the argument of the appreciable military and political costs and risks of maintaining and using chemical weapons?

Most countries have a defence-only policy for chemicals. Are there circum-

stances short of global chemical disarmament in which the United States would gain from such a policy? Relevant when Utgoff wrote, these questions are far more so today, when the Soviet threat has greatly subsided and US chemical weapons policy can spell the difference between success or failure in achieving the support of other nations for an effective chemical disarmament treaty. □

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Protein-rich menu

Richard Virden

Proteins: Form and Function. Edited by Ralph A. Bradshaw and Mary Purton. Elsevier: 1990. Pp. 270. £21.

Protein Engineering: Approaches to the Manipulation of Protein Folding. Edited by Saran A. Narang. Butterworths: 1990. Pp. 262. £55.

THE pioneer's enthusiasm for the application of recombinant DNA techniques to produce useful amounts of valuable proteins is nowadays tempered by the realization that proteins are more than derivative linear polymers. Isolation of a correctly folded recombinant protein is often a non-trivial problem which can only be tackled empirically, drawing on such experience as that gained in the experimental study of protein folding. Intelligent genetic manipulation of protein properties is a yet greater challenge, demanding a thorough understanding of protein conformation, stability and relevant interactions with other molecules. The design of improvements in protein stability by changing individual amino-acid residues is made more difficult by the fine balance of large opposing forces which define the overall, often marginal, stability of the folded state. For the most part, these properties cannot be predicted from amino-acid sequences and therefore must be determined by experiment. Such studies are necessarily broad in scope and they are being developed and applied to an ever increasing number of proteins. Both specialists and newcomers to protein science will therefore welcome collections of up-to-date, authoritative and digestible overviews of these developments; two rather different multi-author menus of protein-rich nourishment are considered here.

The first, *Proteins*, contains 16 articles first published in the July 1989 issue of *Trends in Biochemical Sciences* together with 13 more articles all grouped under four main headings: primary protein

structure; protein conformation; co- and post-translational modifications; and molecular recognition.

The pivotal conformation section appropriately opens with X-ray crystallography as the mainstay of three-dimensional structure determination. There is a comprehensible and forward-looking assessment of the state of the art and well-illustrated summaries of methods of crystallization, data collection and phase determination. A more specialized account of catalytic intermediates in enzyme crystals looks to a future when high-intensity pulsed X-ray sources will permit exposure times as short as 10–100 ps. Nuclear magnetic resonance (NMR) is shown as having a distinctive place in structure determination potentially extending to proteins (and independently folding domains of larger proteins) up to a relative molecular mass of 30,000. There is an excellent introduction to experimental study of the initiation of folding in small, single-domain proteins. This shows how progress is being made towards answering a key question — is there an obligatory order in the processes of hydrophobic collapse, formation of a compact molten globule state, and formation of hydrogen-bonded secondary structure? The aspiring protein engineer, eager for accurate conformational prediction from amino-acid sequences, will find some encouragement for the fashionable practice of modelling membrane-spanning helices and optimism for the prediction of secondary structure in globular proteins. But apart from modelling based on sequence similarity with previously determined structures, success in predicting tertiary structure is evidently some way off, with "unlimited future prospects". On the other hand, progress is reported in the converse problem of designing sequences which will fold in solution to specified conformations.

Earlier chapters include reviews of recent developments in protein and peptide purification and in primary structure analysis, featuring the increasing importance of developments in sensitive techniques such as mass spectrometry. There

is also a recent summary of techniques in site-directed mutagenesis. Other contributions focus on particular proteins and biological processes. Occupying both scientific and industrial high ground are the achievements of site-directed and random mutagenesis in modifying subtilisin in its catalysis, specificity, pH/rate profile and stability to some of the rigours of the wash-tub. There are further accounts of studies based on high-resolution structures, such as site-directed mutagenesis of lactate dehydrogenase and molecular recognition by helix-turn-helix DNA-binding proteins, and there is consideration of less detailed conformational information, for example in relation to the *lac* permease. Chapters on protein translocation, posttranslational modification, proteolytic processing, glycosylation and secretion give a taste of some of the complexities being confronted in identifying the significant molecular interactions of proteins in a cellular context.

Although a significant number of these articles may be found in one issue of *Trends in Biochemical Sciences*, the advantages of the enlarged collection will make this an attractive book for both advanced undergraduates and graduates, giving concise and reasonably self-contained introductions and pointers to the recent literature over a very wide range of current concerns in protein science.

The second book, *Protein Engineering*, comprises 11 chapters, several relating mainly to techniques. There are useful descriptions of a variety of techniques for study of protein folding and quite full descriptions of spectroscopic techniques including X-ray crystallography, NMR, laser-based fluorescence, and infrared spectroscopy for characterization of membrane proteins. How site-directed mutagenesis affects protein folding is the concern of one chapter, taking tryptophan synthase α -subunits and dihydrofolate reductase as major examples. Other contributors discuss ways of identifying functional sites in a protein of unknown conformation (alanine-tRNA synthetase), and epitope mapping of the envelope glycoprotein of the human immunodeficiency virus.

In view of the difficulties of prediction, protein engineers will probably continue for some time to come to be concerned more with the effects of mutation on the final folded conformation than on folding pathways. With its emphasis on folding, this book can therefore be recommended more for its introductions to techniques and for studies on particular proteins than as an overview of mainstream developments in protein engineering. □

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Seismic activity on neighbouring faults as a long-term precursor to large earthquakes in the San Francisco Bay area

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Activity in moderate-size earthquakes accelerated in the several decades before the large California earthquakes of 1989, 1906 and 1868. This type of precursor seems to require the presence of several major faults in close enough proximity to one another that moderate-size shocks are selectively triggered on surrounding faults during the latter stages of the cycle of strain buildup to large earthquakes. It may be possible to use quantitative aspects of similar seismic precursors to make predictions of large earthquakes on timescales of a few years to one decade.

THE number of moderate-size earthquakes in the greater San Francisco Bay area was much higher in the 25 years before the great San Francisco earthquake of 1906 than it was in the longer period from 1912 to 1955^{1,2}. Ellsworth *et al.*² noted the re-emergence of earthquakes of magnitude $M = 5.0$ – 5.9 from 1955 to 1980 after a long quiescence and concluded that the region was entering a more active stage in which events as large as $M = 6$ to perhaps $M = 7$ could be expected in the decades before an eventual great earthquake. Sykes and Nishenko³ remarked that the region of increased activity for the period 1955 to 1982 was not as large as that preceding the 1906 shock and instead was concentrated mainly to the east and southeast of San Francisco. They concluded that the pattern might represent a long-term precursor to an earthquake of magnitude near 7 along that segment of the San Andreas fault from opposite San Jose to San Juan Bautista. The Loma Prieta earthquake of 18 October 1989 of magnitude 7.1, the first large ($M \geq 6.8$) shock to occur along the San Andreas fault itself since 1906 was, in fact, centred along that fault segment.

We find that the activity in moderate-size shocks ($5 \leq M < 6.5$), particularly the seismic moment M_0 , increased in the several decades before the 1989, 1906 and 1868 events, the three largest earthquakes in the San Francisco Bay area during the past 150 years. The accelerated release rates, two of which were terminated by the occurrence of the 1906 and 1868 shocks, resemble tertiary creep. This process occurs in a wide variety of materials including rocks and is characterized by accelerating deformation which leads to failure^{4–6}.

Forerunning activity to large earthquakes

We now examine the spatial and temporal distribution of seismicity before three large earthquakes in the San Francisco Bay area. Figure 1 shows earthquakes of $M \geq 5$ for 35-year periods preceding the 1906 and 1989 earthquakes, the 35-year period 1920–1954, which was well separated in time from a large shock, and the 14 years preceding the 1868 earthquake. The shorter period before the 1868 event reflects an incomplete record of moderate-size shocks. The area of our study is the same as that used by Ellsworth *et al.*² in their examination of long-term forerunning activity to the 1906 earthquake. It is bounded on

the southeast by a segment of the San Andreas fault that shows continuous creep, rather than the stick-slip rupture of large earthquakes. It is unlikely that precursory changes related to large earthquakes in the study area extend into that region. The magnitudes used are from refs 7–11.

We chose to study only earthquakes of $M \geq 5.0$ in Fig. 1 because: (1) catalogues of events of smaller magnitude are not complete before the 1906 and 1868 earthquakes, the only two events in the past 150 years similar to or larger in size than the 1989 shock; (2) the frequency of occurrence of events of $M \geq 5$ is known to have varied greatly before and after the 1906 shock^{1,2}; (3) we did not find a significant variation in the frequency of occurrence of events of $4 \leq M < 5$ for 1942–1989, during which period magnitudes were calculated by the same procedure and it is believed that there was uniform reporting of earthquakes of those sizes^{9,12}.

Figure 1a shows a high level of seismic activity for events of $M \geq 5$ in the San Francisco Bay area in the 35-year period before the great 1906 earthquake. Most, and perhaps all of those shocks, the largest of which was $M = 6.4$, occurred well away from the surface trace of the 1906 fault break. The magnitudes of most of those events are, in fact, larger than those of the aftershocks of the 1906 event. By 1912 activity in earthquakes of $M \geq 5$ had decreased to a very low level. As can be seen in Fig. 1b, no events of $M \geq 5$ occurred in the study area to the north of 37.0° from 1920 to 1954. Seismic activity in shocks of $M \geq 5.0$ was also high before the 1989 and 1868 earthquakes (Fig. 1c, d). Activity dropped to a very low level within two years of the 1868 shock (Fig. 1d); no events of $M \geq 5$ are known to have occurred in the area of Fig. 1 between 1871 and 1881. The low release rate is similar to that from 1912 to 1954, following the great 1906 earthquake. Ellsworth *et al.*² concluded that changes in the frequency of occurrence of events of $M \geq 5$ in the vicinity of the 1906 fault break from 1855 to 1980 were significant at the 95% confidence level.

As in Fig. 1a, the activity preceding the 1989 event (Fig. 1c) occurred on faults other than the one that broke in the 1989 earthquake. Even the two Lake Elsin shocks of June 1988 and August 1989 ($M = 5.0$ and $M = 5.2$), which occurred close to the epicentre of the 1989 earthquake and can be considered to be long-term foreshocks, were situated a few kilometres away from the main rupture zone of the Loma Prieta shock, on faults dipping in the opposite direction to the one that ruptured in the 1989 event¹³.

Accelerating seismic moment release

Ellsworth *et al.*² remark that it is paradoxical that the production rate of smaller earthquakes in the Bay area remained virtually constant (the record is complete since 1930 for $M \geq 4$ and since 1942 for $M \geq 3$), in contrast to marked variations in activity for $M \geq 5$. Recognizing that the changes in seismic activity with time in Fig. 1 are also largely confined to shocks of $M \geq 5$, we measure changes in activity in terms of a temporal sum of the seismic moment, regarding this as a first-order approximation to the regional change in shear strain associated with earthquakes¹⁴. The principal contribution to the sum comes from the several largest shocks in a given sample; the smallest events

contribute very little. Hence, an increase in seismic moment with time is consistent with the observation that the number of events of $M \geq 5$ increased before large shocks whereas the rate of occurrence of smaller shocks in the same broad area remained nearly constant. Unlike counts of numbers of earthquakes, the moment sum is relatively insensitive to changes in detection levels.

We use cumulative seismic moment ΣM_0 to quantify moment release, where M_0 is summed from a given date for shocks of $M \geq 5.0$ that occurred within the precursory areas of Fig. 1, which we describe below. As the release of seismic moment occurs virtually instantaneously during earthquakes, we prefer to sum moment release with time rather than work with moment rate. All of the seismic moments used were calculated¹⁵ from $\log_{10} M_0 = 1.5M + 9.0$, where M is the local magnitude reported by the Berkeley seismographic station for events in the Bay area^{9,10}. Moment has also been determined independently from long-period waves for most events of $M \geq 5.3$ in the study area since 1977¹⁶. The two determinations are in excellent agreement, indicating no significant biases in the calculation of moment since 1977.

Figure 2a shows ΣM_0 in the San Francisco Bay area as a function of time since 1955. The upward curvature indicates an increase in the rate of moment release (the slope) from 1955 to 1989. ΣM_0 increased much more rapidly from 1979 to 1988 than it did during previous decades. Much of that release is contributed by the 1979, 1980, 1984 and 1986 earthquakes (Fig. 1c). The rise time τ of the best-fitting exponential is 11 years. This function fits the data with a root-mean-square uncertainty that is $\sim 50\%$ smaller than that for the best-fitting linear increase in ΣM_0 ; this is also true for the three other data sets we examined.

Values of ΣM_0 for the periods leading up to both the 1906 and 1868 earthquakes are shown in Fig. 2b, d. A very low rate

of moment release from 1872 to 1881 is followed by an accelerating rate. A similar effect is seen in the 14 years preceding the 1868 earthquake. The rise time of the best-fitting exponential for the pre-1906 sequence is 9 years. That for the pre-1868 data, 4 years, is poorly determined and is probably too small because it is based on only 14 years of data.

Can the accelerated releases of moment before the three large shocks be explained instead by a systematic error in the calculation of M_0 as a function of time? When M_0 is derived from M , ΣM_0 is sensitive to systematic changes in magnitude determination, which would produce an apparent change in the rate of moment release. We examined events of $M \geq 4.0$ from 1942 to 1989 for such changes using the techniques of Habermann¹⁷ but found no evidence for a systematic magnitude change. For the decade before the 1989 event, ΣM_0 is 6.3 times larger than that for the preceding decade (Fig. 2a). An error in the magnitude of 0.5 is required to account for that difference, which seems unlikely because such a large systematic error is not indicated for events of $M \geq 4.0$. Also, it would be a very unlikely coincidence for large biases in M to occur such that they slowly increased before the 1868, 1906 and 1989 events and then suddenly decreased in the several years after the 1868 and 1906 earthquakes. More work is needed to obtain a better calibration of M_0 as a function of intensity for modern earthquakes and then to use it to derive moment for pre-instrumental earthquakes such as those before 1906 for which M was estimated from the area over which the earthquakes were felt.

Removal of the largest event from 1955 to 1989, the 1984 earthquake, results in linear and exponential relationships with comparable misfits, an indication of the sensitivity of ΣM_0 to the inclusion or exclusion of the largest events in a sample. In that case, however, the average yearly rate of moment release from 1955 to 1989 in the precursory area of the 1989 event is still six times that from 1912 to 1954.

FIG. 1 Distribution of earthquakes of magnitude 5 or larger in San Francisco Bay area for four time intervals. Major faults are shown; epicentres of 1906, 1989 and 1868 earthquakes indicated by large solid circles. Locations of earthquakes and extent of rupture in three large shocks (heavy solid lines) are from refs 2 and 7–11. Arrows in *a* denote sense of strike-slip motion along the San Andreas fault. Dashes enclose events taken to be within the precursory area of the 1906, 1989 and 1868 events. The activity was low in the precursory area of the 1989 shock (dashes) in *b* from 1920 to 1954.

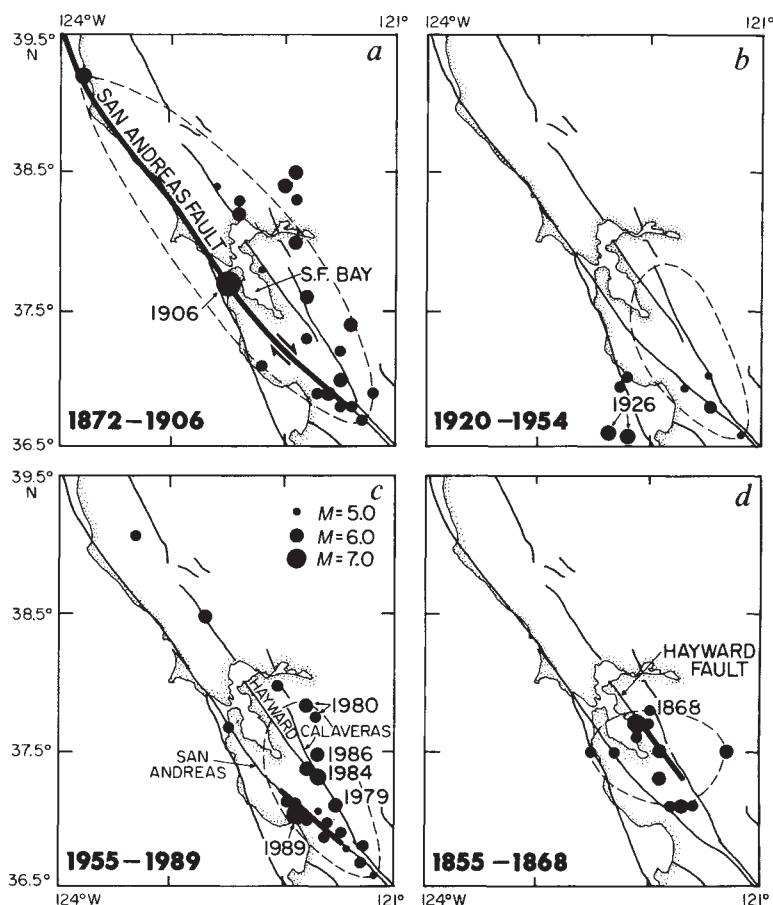
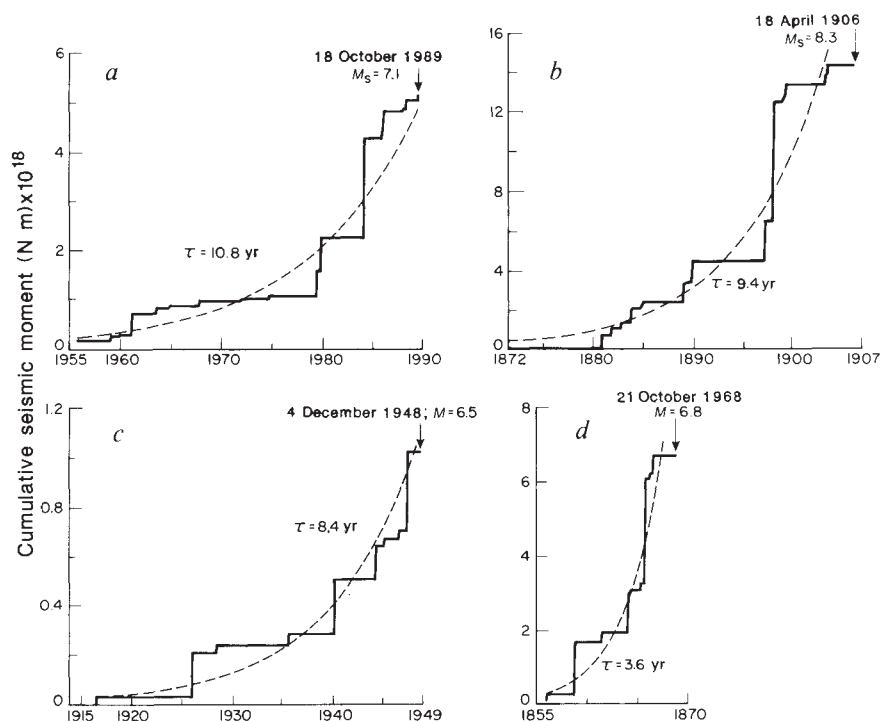


FIG. 2 Cumulative seismic moment ΣM_0 summed for events of $M \geq 5.0$ as a function of time t before three earthquakes in San Francisco Bay area and the 1948 shock in southern California. Dashed lines are the best-fitting exponential increases, $\Sigma M_0(t) = \Sigma M_0(0)e^{t/\tau}$, where τ is rise time.



Other examples of precursory moment release

The rate of occurrence of shocks of $5.0 \leq M < 6.0$ within 50 km of the epicentre of Desert Hot Springs earthquake of magnitude 6.5 in 1948 in southern California increased by a factor of 10 in the preceding 8.5 years^{18,19}. For the 35-year period before that shock, ΣM_0 , as calculated from the local magnitude, increased nearly exponentially with a rise time of 8 years (Fig. 2c). ΣM_0 for each of the four precursory sequences in Fig. 2 can be fit with functions other than increasing exponentials. Good fits to the data, however, seem to be restricted to functions that yield higher rates of moment release in the one or two decades before the large shocks and lower rates in the preceding few decades. None of the aftershocks of the 1948 earthquake exceeded $M = 4.9$, and none of the forerunning events occurred in its rupture zone (as deduced from the distribution of aftershocks)¹⁸⁻²⁰. Thus, the 1948 event was very similar to the 1906 and 1989 earthquakes in having a large number of moderate-size events preceding it in a broad surrounding area.

The descriptions by Mogi²¹ of doughnut-shaped patterns of $M \geq 6.0$ earthquakes surrounding the rupture zones of several great earthquakes at subduction zones are qualitatively similar in space and time to the patterns we report. Much of that forerunning activity occurred in the few decades before the great thrust events, either in adjacent portions of the upper plate or on nearby segments of the plate boundary along the strike.

Determination of precursory area

We have identified the regions that we think participated in the precursory release of seismic moment to three large earthquakes

(Fig. 1, dashed lines) and have estimated their precursory area A (Table 1). More-quantitative techniques need to be applied to determine A more objectively and accurately in future studies. Activity in the 35-year period before the 1906 earthquake (Fig. 1a) extended along much of the 430-km length of the 1906 rupture zone. Only one event is taken to define the northern extent of A for that shock. Because it is one of the larger events in Fig. 1a and because it occurred only 8 years before 1906, we chose to include it in our definition of A . Shocks of $M \leq 5.5$ from 1872 to 1906 may not have been reported from sparsely populated areas north of 39°N . We also exclude from A the 1892 events that are farthest (80–100 km) from the 1906 rupture zone. Deformation in that area seems to be the thrust type, like that farther south along strike near Coalinga²². Inclusion of these events results in a poorer fit to a rising exponential in Fig. 2b but an even higher rate of precursory moment release.

Most of the forerunning activity to the 1989 shock (Fig. 1c), especially the larger events, occurred south of 38.0°N . Except for the two events in 1980, all of the shocks to the north of 37.5° occurred 20–35 years before the 1989 earthquake. Hence, those early events may not be part of the preparatory process of the 1989 event and were not included in our estimate of A . Judging from the distribution of newspapers in print, the basis for identifying and locating most of the events before 1906^{7,8}, the record of earthquakes of $M = 5-5.5$ in Fig. 1d may not be complete south of 37.0° or north of 38.5°N before 1860. Although forerunning activity to the 1868 event may have occurred farther south than indicated in Fig. 1d, the other boundaries of its

TABLE 1 Data on precursory sequences

Precursory sequence	Magnitude of mainshock	Precursory Area ($\text{m}^2 \times 10^9$)	Rise time, τ (yr)	Accumulated seismic moment ΣM_0 just before main event ($\text{N m} \times 10^{18}$)	Equivalent magnitude of ΣM_0	$\Sigma M_0/A$ ($\text{N m}^{-1} \times 10^8$)	$M_0/A = \Sigma M_0/A\tau$ ($[\text{N m}^{-1} \text{yr}^{-1}] \times 10^8$)
1872–1906	8.3*	22.0	9.4	14.3	6.8	6.5	0.69
1955–1989	7.1*	7.1	10.8	5.2	6.5	7.3	0.68
1855–1868	6.8	6.8	(3.6)	6.7	6.6	9.9	(2.7)
1914–1948	6.5	2.2	8.4	1.03	6.0	4.7	0.56

* M_s , surface-wave magnitude.

precursory area are not likely to be affected by the incompleteness of reports.

We chose to sum M_0 over a 35-year period for the four earthquakes we studied based on the data of the 1989 shock and the finding in ref. 2 that $M \geq 5$ events became more numerous after 1955. Shortening the summation interval to 25 years, the time period for which shocks of that size became more numerous before the 1906 earthquake, affects ΣM_0 very little, especially for the last decade of precursory release. Taking a longer time interval mainly affects the determination of A because events are included that may not be part of the precursory process.

We distinguish separate precursory sequences for the 1868 and 1906 mainshocks. From the temporal sequences alone, it is possible that the period of low activity from 1872 to 1881 occurred by chance and that the two precursory sequences are, in fact, one. The precursors we report, however, have spatial characteristics as well. The precursory areas and the magnitudes of those two mainshocks are quite different in size, providing strong evidence that the two events have distinguishable precursors. Although our determinations of A are considerably larger than the aftershock areas of major earthquakes, they are much smaller than estimates of A used in ref. 23.

Mechanism of precursory moment release

The region within about 50–75 km of each rupture zone contains several major active faults in addition to the fault that ruptured in each mainshock of Fig. 1. Several of the larger events that preceded the 1989 earthquake occurred along the southern Calaveras fault within 50 km of the 1989 rupture zone (Fig. 1c). Segments of several nearby faults that were already close to rupturing in moderate-size events seem to have moved more rapidly toward failure during the several decades before the shock. The locations and focal mechanisms of the moderate-size shocks in Fig. 1c and the 1989 event are in accord with the idea that strain buildup to the 1989 earthquake could have augmented the strain that was otherwise building up along the adjacent faults.

Figure 3 shows schematically our conception of the release of seismic moment as a function of space and time in the San Francisco Bay area and in the area of the 1948 shock. Our ideas expand earlier work on the seismic cycle of large earthquakes²⁴. After the occurrence of a large earthquake and its aftershock sequence (Fig. 3b), strain is built up slowly in the area surrounding the main rupture zone. During the latter stages of that buildup, moderate-size earthquakes are preferentially triggered on various fault segments in the surrounding precursory area (Fig. 3a, c). The rate of moment release in the area accelerates as the time of the next large shock approaches (Fig. 3c). Dislocation models of candidate large earthquakes can be used to ascertain which nearby fault segments are expected to move closer to failure and which are not. Changes in the strain associated with moment release in a broad area, which appear to be akin to the non-elastic process of tertiary creep, should not be equated with strain buildup measured geodetically, which seems to be mainly an elastic process.

Mogi²¹ found that forerunning seismic activity in fracture experiments in the laboratory was prevalent for heterogeneous materials and either infrequent or absent for homogeneous materials. By analogy, he proposed that large-scale structural heterogeneity in seismically active regions would be associated with seismic precursors whereas homogeneity would not. We expect that areas with only one major throughgoing fault, such as the Carrizo Plain segment of the San Andreas fault, to be typified by few if any seismic precursors of $M \geq 5$. Many seismically active areas, including the Bay area and large parts of southern California, however, are characterized by a greater complexity in the distribution of active faults, a situation that is commonly thought to make earthquake prediction very difficult. The type of seismic precursor we describe, however,

seems to require a certain amount of tectonic complexity for its existence.

Dislocation modelling of geodetic data indicates that slip at the time of the 1989 earthquake extended to a depth of ~ 18 km (ref. 11). Significant changes in strain are likely to have occurred to distances a few times 18 km, both during the 1989 event and in the long period of strain buildup before it. The sizes of the precursory areas deduced for the 1868 and 1989 events (Fig. 1) seem reasonably attributed to such a process. Most of the forerunning activity to the 1906 and 1989 shocks (Fig. 1) is located asymmetrically with respect to the San Andreas fault as is the zone of strain accumulation of the past 20 years²⁵. The asymmetrical patterns may result from the main plate boundary at depth being displaced to the northeast of the San Andreas fault²⁶. The 1948 shock occurred in a similar setting²⁷, suggesting that this type of precursor may occur at greater distances when the plate boundary is not vertical throughout the lithosphere.

Predicting large earthquakes in the Bay area

Our work raises some questions. Did the 1989 shock terminate a sequence of accelerated or high moment release? When and where will the next large, $M \geq 6.8$, earthquake occur in the San Francisco Bay area? What are the uncertainties in such estimates? Are all large shocks in the region preceded by similar anomalies?

The 1868 and 1906 earthquakes were followed by long periods of low moment release and small numbers of events of $M > 5.0$ (Figs 1 and 2). Thus, they culminate as well as terminate the long-term increase in the rate of moment release. Using the patterns of moment release in the years to decades following the 1868 earthquake as a guide to what may happen on similar timescales after the 1989 event, which was of comparable size, we foresee three possibilities for future large earthquakes in the Bay area. If, within several years, the rate of moment release and the rate of occurrence of events of $M \geq 5.0$ drop to their

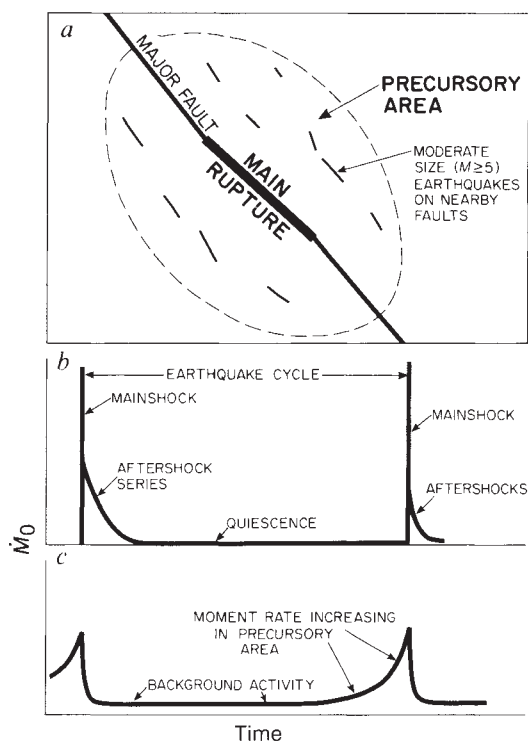


FIG. 3 Schematic diagrams showing relationship of seismic precursors to cycle of strain buildup and release in large earthquakes in the San Francisco Bay area. *a*, Map view of moderate-size shocks in the precursory area surrounding the rupture zone of the coming large earthquake. Rate of release of seismic moment as a function of time along *b*, the main rupture zone itself and *c*, in the precursory area.

low 1912–1954 levels, a large shock is unlikely to occur for at least a decade and then only when either ΣM_0 or the rate of moment release increases to values comparable to those just before the 1989 earthquake. But if it becomes evident with the next few years that those rates have remained high, a second large event can be expected. The third possibility is that a second large earthquake could occur within a few years, but after a period not long enough for a good determination of the rates.

Our experience with seismic precursors to large shocks in the Bay area comes from the southern half of the Hayward fault and from nearly the entire portion of the San Andreas fault in Fig. 1. It seems reasonable to assume that future large earthquakes along these fault segments will be preceded by similar anomalies. As the northern Hayward fault, last broken in the large event in 1836⁸, is partly surrounded by faults that ruptured in moderate-size events before the 1906 shock, it seems likely that a repeat of the 1836 shock would also be preceded by a pattern like that before the 1868 event but centred around the northern half of the fault. Not very much of the activity in Fig. 1c is situated within 50 km of that segment, however, in contrast to that in the decades before the 1868 and 1989 earthquakes. Two of the six events that occurred within that distance between 1955 and 1989 occurred over 33 years ago. If a large event were imminent along the northern Hayward fault in the next few years, it seems likely that greater nearby activity would have occurred already. Likewise, there is no indication of a buildup of activity surrounding the San Andreas fault to the north of San Francisco, supporting the idea that there is a low probability of that fault segment rupturing in a large to great shock in the next few decades³.

The situation for the southern Hayward fault and for the San Andreas fault between San Francisco and the 1989 rupture zone is more equivocal because $M \geq 5$ events of the past 30 years within 50 km of these segments could be interpreted as precursors of either the 1989 shock alone or of both it and a future large earthquake along one of those segments. The amount of damage and loss of life in a shock of about $M = 7$ along either the Hayward fault or along the peninsular section of the San Andreas fault, when it occurs, are likely to be considerably larger than those in the 1989 earthquake. Hence, although such an event would be smaller than the 1906 earthquake, it is likely to be called 'the big one' by the public.

There are several ways in which information on moment release might be used quantitatively in making predictions of future large earthquakes in the study area. For example, ΣM_0 can be thought of as a seismic stress gauge indicating the likelihood of a future large earthquake in the Bay area. The values of ΣM_0 just before the 1989 and 1868 events are very similar (Table 1). If other considerations, such as the dimensions of a candidate fault segment, indicate that a future earthquake in the Bay area is likely to be of magnitude near 7, then the experience from the 1868 and 1989 shocks indicates that the

mainshock is likely to occur within a few years of the time when ΣM_0 reaches 6×10^{18} N m.

Table 1 shows values of ΣM_0 just before the three large shocks in the study area and the 1948 earthquake. Both ΣM_0 and the precursory area increase as the size of the mainshock increases. We normalize for this effect by dividing ΣM_0 and its time derivative $\dot{M}_0$, by A . The values of $\Sigma M_0/A$ in Table 1 are similar; their mean and standard deviation are $(7.1 \pm 2.2) \times 10^8$ N m⁻¹. Using $\tau = 10.8$ years from the 1955–1989 sequence, one standard deviation translates into an uncertainty in predicting the time to failure T_f (the time required to accumulate 2.2×10^8 N m⁻¹ when $\Sigma M_0/A$ is close to the mean value) of about three years. A smaller uncertainty in T_f would be associated with smaller values of τ in Table 1. Values of $\dot{M}_0/A$ before each mainshock (Table 1) exhibit less scatter than values of $\Sigma M_0/A$, providing the more uncertain rate for the pre-1868 sequence is omitted. Thus, the levels of $\Sigma M_0/A$ and $\dot{M}_0/A$ may be useful in predicting future large earthquakes to within a few years for regions of similar precursory release.

Discussion

Voight⁵ describes rate-dependent failure in a variety of materials by the empirical equation

$$\ddot{\Omega} - A\dot{\Omega}^\alpha = 0 \quad (1)$$

where A and α are constants, and $\ddot{\Omega}$ and $\dot{\Omega}$ are the first and second time derivatives of a measure of the deformation process. For $\alpha = 1$, Ω grows exponentially and $A = \tau^{-1}$. For $1 < \alpha < 2$, Ω becomes very large at a finite time to failure. For a variety of materials⁵ and for foreshocks²⁸, α is typically somewhat smaller than two. Equating ΣM_0 and Ω in equation (1) and taking ΣM_0 to include M_0 for the mainshock at its known time of occurrence, we obtain a best fit to the data for the precursory sequences of the 1948 and 1989 events for similar values of α . The similarity in α for these phenomena suggests a similarity in the associated failure processes. We have not been successful, however, in making accurate estimates of T_f from ΣM_0 using equation (1).

Each of the patterns of precursory seismicity we have described involves an accelerated release of seismic moment with time and a clustering of activity in the region surrounding the coming large event. The similarity of the sequences in time and space before three large earthquakes in the Bay area and the 1948 shock argues that these patterns are causally related to the four mainshocks. We are optimistic that large earthquakes preceded by this type of precursor will be predictable with greater accuracy. The number of examples, however, is still too small to ascertain the reliability of the predictions.

Note added in proof: High rates of moderate-size earthquakes that are similar to the patterns we report occurred in the decades before the large shocks of 1703 and 1923 in Japan²⁹, 1952 in southern California³⁰, and 1957 in the central Aleutian Islands³¹. □

Received 17 May; accepted 20 November 1990.

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ACKNOWLEDGEMENTS. We thank R. E. Habermann, A. G. Lindh, W. Menke, C. H. Scholz and D. W. Simpson for reviews and M. R. McKenzie for furnishing magnitudes from the Berkeley network for events in 1989. This work was supported by the NSF and the U.S. Geological Survey.

Intracellular transport of class II MHC molecules directed by invariant chain

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Three structural motifs in the invariant chain (Ii) control the intracellular transport of class II major histocompatibility complex molecules. An endoplasmic reticulum retention signal in the full-length Ii suggests a role for Ii in the α - β heterodimer assembly. Another signal motif directs a truncated Ii, alone or associated with individual class II chains, to a degradation compartment by a pathway circumventing the Golgi. When this truncated Ii binds α - β dimers, a third signal dominates, directing the complex by way of the Golgi to vesicles in the cell periphery, which may represent a subcompartment of recycling endosomes.

CLASS I major histocompatibility complex (MHC) molecules acquire peptides from endogenously synthesized antigens, whereas class II MHC molecules present exogenously derived peptides. Class II molecules associate with an invariant chain (Ii) in the endoplasmic reticulum (ER)^{1,2}, preventing the binding of endogenous peptides^{3,4}. To bind to peptides, class II molecules have to divest themselves of Ii. The dissociation occurs in an acidic cellular compartment and is accompanied by proteolysis of Ii^{5,6}. Previous reports have suggested that Ii is involved in the intracellular transport of class II molecules⁷⁻⁹. But transfected cells expressing only class II α and β chains transport the assembled molecules to the cell surface so that they are functional^{8,10}.

Transport of proteins in the exocytotic pathway occurs by default^{11,12}. Deviation from the default pathway depends on structural motifs in the transported proteins^{13,14}. Therefore, either the class II α and β chains or Ii must have the targeting signal that directs the complex to the intracellular compartment where Ii dissociates from the α - β heterodimer. In expressing class II α and β chains alone or with Ii we have now demonstrated that Ii has three structural motifs directing its intracellular distribution.

Class II α and β chains lack targeting signals

To investigate whether class II molecules expressed in the absence of Ii have the same subcellular distribution as class II molecules expressed in the presence of Ii, we examined transfected HeLa cells by indirect immunofluorescence staining. Permeabilized, transfected cells expressing class II α and β chains in the absence of Ii stained uniformly for the heterodimer at the cell surface (Fig. 1). Strong staining occurred in the perinuclear area, coincident with that for wheat-germ agglutinin (not shown), suggesting that it represented the concentration of class II molecules in transit through the Golgi complex. For class II molecules expressed with Ii, the staining was punctate in the central portion of the cell (Fig. 1b). The distribution of

Ii was similar but more prominent in the periphery of the cell (Fig. 1c). Fluorescence-activated cell sorting demonstrated that the transfectants with and without Ii expressed similar amounts of class II molecules at the cell surface, and biosynthetic pulse-chase experiments showed that the class II α and β chains were resistant to endoglycosidase H (endo H) with similar kinetics in both transfectants (not shown). These data confirm that class II α and β chains can assemble and be transported to the cell surface without Ii⁸. But Ii seems to direct the class II molecules to a novel compartment. To identify this compartment, HeLa cells expressing class II α and β chains and Ii were separately stained for class II molecules, the transferrin receptor (a marker for early endosomes)¹⁵, the mannose-6-phosphate receptor (M6PR) (a marker for late endosomes)¹⁶ and the lysosomal protein LampA¹⁷. Figure 1d and e show a partial overlap in the staining of class II molecules and M6PR, suggesting that some class II molecules are present in late endosomes. Most vesicles containing the LampA protein lacked class II molecules. Likewise, the subcellular distributions of the transferrin receptor and the class II molecules were different (not shown).

Intracellular transport of Ii mutants

Ii has several forms. Four translation products of human Ii arise from two separate messenger RNAs, which differ by an alternatively spliced exon¹⁸⁻²⁰. Two separately used initiation codons give polypeptides differing in length by 15 amino-acid residues^{19,20}. The translation products are modified by the addition of *N*-linked and *O*-linked carbohydrate moieties²¹ and minor forms of Ii are acylated²², sulphated²³, phosphorylated²⁴ or contain glycosaminoglycans²⁵.

Transport signals seem to consist of short sequences of amino acids rather than post-translational modifications¹¹⁻¹³. Ii is a type II transmembrane protein²⁶, so its cytoplasmically exposed N-terminal region might contain transport signals. To identify such signals we generated a series of deletion-mutant constructs of a full-length complementary DNA clone encoding human Ii (Fig. 2a) and inserted them into an expression vector²⁷. As the full-length cDNA has two alternatively used initiation codons^{19,20}, we changed the second methionine codon to that for isoleucine and optimized the Kozak consensus sequence so that only the full-length protein (Iip33) could be expressed. To ascertain the expression from the second ATG codon, the cDNA clone was truncated to eliminate the first ATG codon (Iip31). Further deletions involved the N-terminal 9, 16 and 29 residues, respectively, of Iip31 (to give Iip31D9, Iip31D16 and Iip31D29; (see Fig. 2a)).

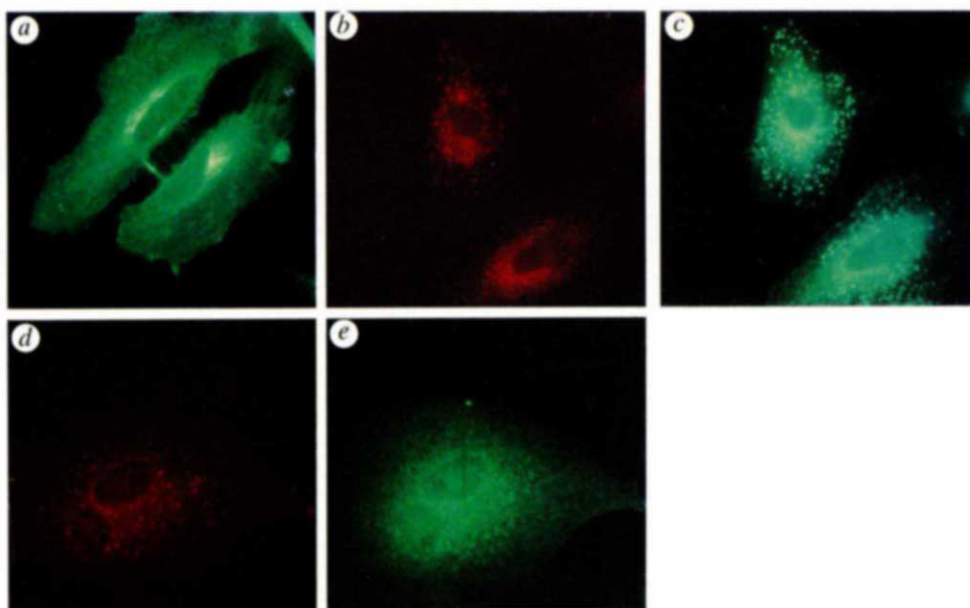
Each of the five different constructs was transfected into HeLa cells, which do not express measurable levels of endogenous Ii. The intracellular transport of the various forms of Ii was examined by biosynthetic pulse-chase experiments. Radiolabelled Ii was immunoprecipitated and analysed by SDS-PAGE. To identify molecules exported out of the ER, portions of the immunoprecipitates were treated with endo H. Resistance to the enzyme signifies that *N*-linked carbohydrate moieties have become processed in the Golgi compartment²⁸. Figure 2b summarizes the results. Iip33 gave three closely spaced bands of the expected relative molecular mass (M_r). The additional component with an apparent M_r of 25,000 (25K) represents the

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FIG. 1 Immunofluorescence staining of class II heterodimers and Ii in HeLa cells expressing class II α and β chains (a) and the class II chains together with Iip31 (b-e). Antibodies specific for class II heterodimers (a, b, d) and Ii (c) and mannose-6-phosphate receptors (e) were used. Co-staining of the same cells are shown in b and c, and in d and e.

METHODS. HeLa cells were transiently transfected with the indicated constructs. The cells were trypsinized at 48 h after transfection and grown overnight on coverslips. After washing with PBS the cells were fixed with 4% paraformaldehyde in PBS for 20 min, washed with PBS, and incubated with 50 mM NH_4Cl for 10 min. After washes with PBS, the cells were permeabilized with 0.1% Triton X-100 for 3 min and washed with PBS/0.2% gelatin for 15 min. The cells were incubated with the primary antibodies for 30 min in PBS 0.2% gelatin, washed and incubated with the secondary antibodies for 30 min. Fluorescence microscopy was performed using a Zeiss axiophot microscope. Class II molecules were stained with the heteromer-specific monoclonal antibody D1-12 (ref. 47). In a, FITC-labelled antibodies against mouse IgG were used as the secondary reagent. For the co-stainings the cells were incubated simultaneously with



a rabbit anti-bovine 300K M6PR serum cross-reacting with the human homologue or with the rabbit anti-IiK2 serum and the murine monoclonal antibody. FITC-labelled anti-rabbit IgG and Texas red-labelled anti-mouse IgG antibodies were used in the second step.

intraluminal domain of Ii released by proteolysis in the ER and it accompanies all wild-type and truncated forms of Ii. Although the 25K molecule lacks the transmembrane segment, it associates with Ii anchored to the membrane (unpublished data).

The heterogeneity of Iip33 reflects several post-translational modifications, but all species of Iip33 were sensitive to endo-H

throughout the chase period. Iip31 showed less electrophoretic heterogeneity but was also almost completely sensitive to the enzymatic treatment. These data suggest that neither the carbohydrate moieties of Iip33 nor those of Iip31 were processed by Golgi glycosidases. Iip33 is different from Iip31, however inasmuch as its half-life was considerably longer. Like Iip31,

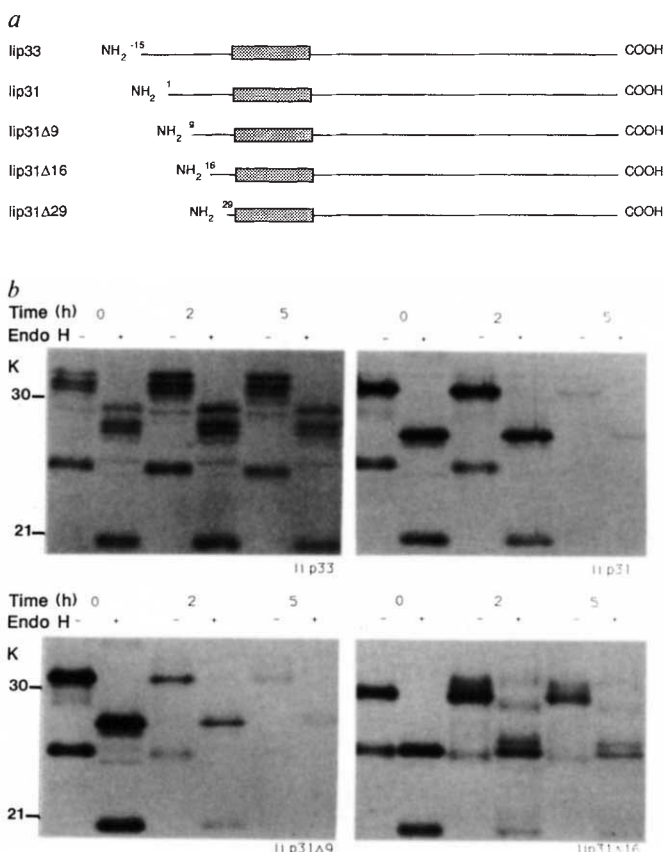
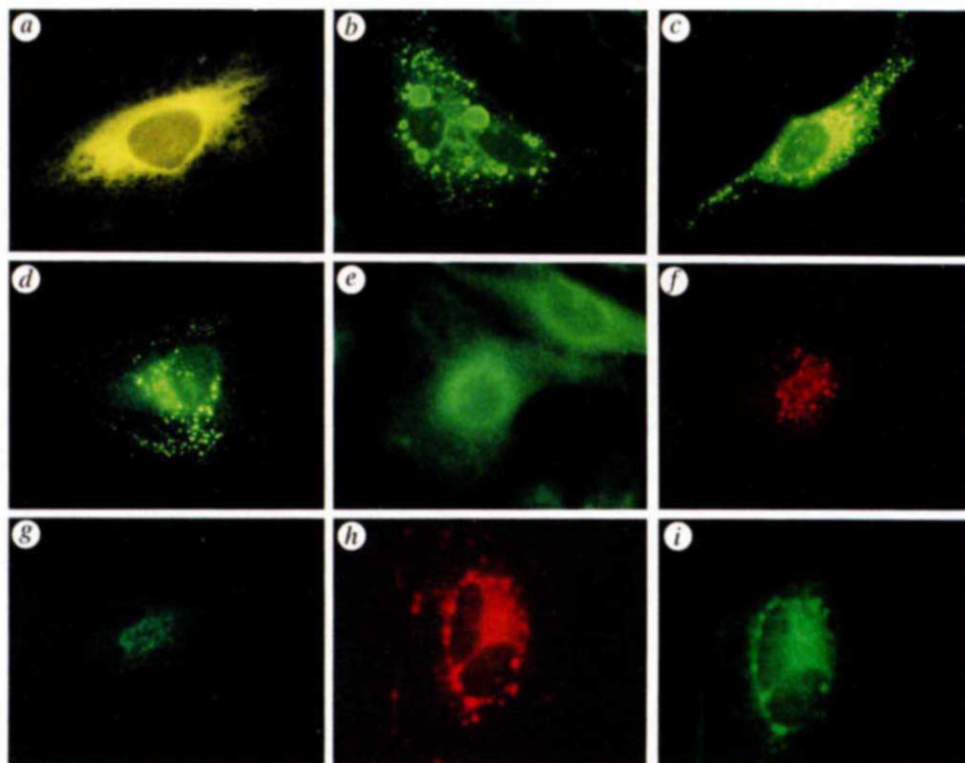


FIG. 2 a, Schematic diagram of wild-type Ii and the deletion mutants. The box denotes the transmembrane segment. The N terminus is to the left. b, Biosynthetic pulse-chase experiments of wild-type Ii and two deletion mutants. Ii was immunoprecipitated at the indicated chase times and subjected to SDS-PAGE with (+) and without (-) prior endo-H treatment. **METHODS.** a, Full-length Ii cDNA obtained as a *Bam*HI fragment was used to generate the deletion mutants. This cDNA contains the two translation-initiation codons. The ATG used to produce Iip31 encodes the first amino acid and the ATG giving rise to Iip33 is codon 15. A *Sal*I cloning site was introduced into the cDNA at codon 19 without changing the encoded amino acids (GGCGCC was changed to GTCGAC). This was accomplished by removing a *Bam*HI-SalI fragment from the cDNA and replacing it with an oligonucleotide that contained an *Xba*I site, the Kozak consensus sequence, ATG and the sequence encoding residues 17-29. The sequence of the oligonucleotide is 5'-GATCCTCTAGACCATGCTGGGCGCTGACCTGGGGCCCGAGAGCAAGTGCAGCCG-3'. The Ii mutant expressed from the modified cDNA is called Iip31Δ16. The mutant Iip31Δ9 was generated by replacing the *Xba*I-SalI fragment in Iip31Δ16 with an oligonucleotide that encodes residues 10-19 preceded by ATG. To construct the Iip31 cDNA the same procedure was used. In this case the oligonucleotide contained a *Bcl*I site at the third codon. This site was used for the cloning of an oligonucleotide encoding residues -15-+3. After sequencing, the modified cDNAs were cloned into the *Bam*HI site of the expression vector pCMU4 (ref. 27). b, HeLa cells were grown in DMEM medium supplemented with 8% FCS, penicillin and streptomycin at $100 \mu\text{g ml}^{-1}$. Transfections by the calcium phosphate technique were carried out as described⁴⁸ except that $20 \mu\text{g}$ DNA and 1×10^5 cells were used per transfection. Metabolic labelling was carried out⁴⁹ with the following modifications. Cells were grown for 72 h after the transfection and [35S]methionine was added to a final concentration of 0.20 mCi in 1 ml of methionine-free medium. After 15 min the cells were chased for various times in the presence of normal culture medium. Immunoprecipitation and SDS-PAGE were carried out as described⁴⁸. After electrophoresis, gels were fixed and treated with Amplify (Amersham). Gradient gels of 10 to 15% acrylamide were used. The K2 peptide rabbit antiserum was used to immunoprecipitate Ii.

FIG. 3 Immunofluorescence staining of Ii in HeLa cells transfected with Iip33 (a), Iip31 (b), Iip31 Δ 9 (c), Iip31 Δ 16 (d) and Iip31 Δ 29 (e). In separate experiments, cells were transfected with Iip31 (f, h) and co-stained with antibodies against M6PR (g) and LampA (i). Immunofluorescence microscopy was carried out as in Fig. 1. Ii was detected by using the rabbit antibody K2 (a–e) and the monoclonal antibody VIC-Y1 (ref. 50) (f, h). Rabbit antibodies against M6PR and LampA were used.



Iip31D9 had a short half-life and was fully sensitive to endo H (Fig. 2b). By contrast, Iip31D16 was only slowly degraded and a portion of this Ii deletion mutant was resistant to endo H (Fig. 2b). Iip31D29 gave rise to two forms of Ii. The most abundant species was secreted from the cell and became fully resistant to endo H (ref. 3). The minor component had an electrophoretic mobility consistent with its retaining the transmembrane segment. As this form of Iip31D29 was absent from the culture medium it seemed likely that it remained attached to membranes. This notion was supported by the solubility of the minor component in the detergent Triton X-114 (ref. 29) and by its long half-life. Like the secreted form, this species also became resistant to endo H.

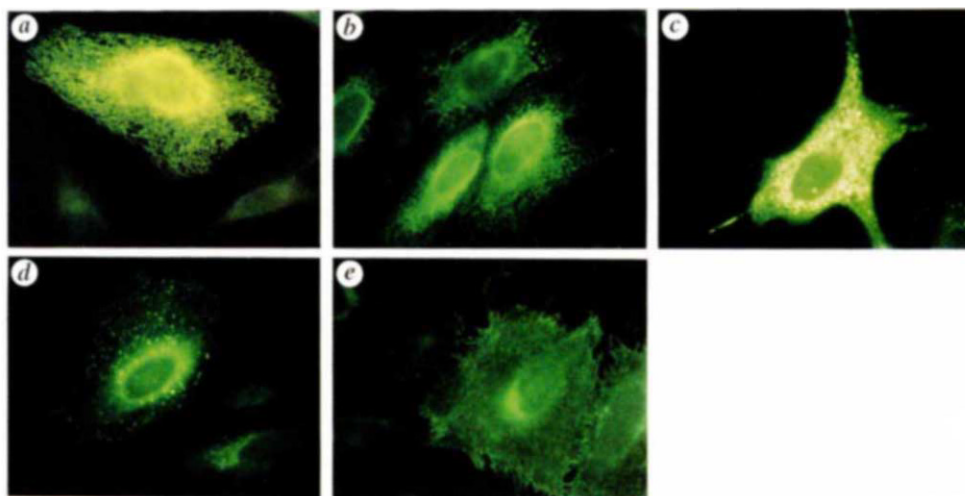
Signals for ER and endosomes/lysosomes

Progressive deletions of the Ii cytoplasmic tail induced carbohydrate processing by Golgi enzymes suggesting that Iip31D16 and Iip31D29 are transported out of the ER. But other forms

of Ii might also be transported despite lacking processed carbohydrate moieties. The longer forms of Ii might have a conformation that impedes carbohydrate modifications (see ref. 49). Alternatively, those forms of Ii might have been routed through the cell such that they escaped contact with the processing enzymes in the Golgi. To study further the transport of Ii, we directly examined the subcellular localization of the various wild-type and mutant proteins by immunofluorescence microscopy and FACS analyses of HeLa cells transfected with the various Ii constructs.

FACS revealed that small but significant amounts of all forms of Ii except Iip33 were present on the surface of the transfected cells, whereas non-transfected HeLa cells completely lacked molecules reactive with the antibodies (not shown). The intracellular distribution was dramatically different for the various forms of Ii (Fig. 3). Cells transfected with Iip33 displayed a perinuclear, reticulate staining which is consistent with the molecule being retained in the ER (Fig. 3a). Co-transfected

FIG. 5 Subcellular distribution of HLA-DR α chains alone (a), or co-expressed with Iip33 (b), Iip31 (c), Iip31 Δ 16 (d), and Iip31 Δ 29 (e) in transiently transfected HeLa cells. A monoclonal antibody against HLA-DR α chains was used⁵¹. Procedures were the same as those described in Fig. 1.



cells expressing lip33 and an ER marker protein, CD8/E19 (ref. 27), were coincidentally stained for the two proteins. We thus conclude that lip33 is confined to the ER.

Cells expressing lip31 gave a different staining pattern (Fig. 3b). The diffuse perinuclear fluorescence was greatly diminished and a weak but significant staining over the whole cell body confirmed that this form of li was expressed at the cell surface. But most of the lip31 gave rise to a punctate staining pattern. Small vesicle-like structures were predominant in the periphery of the cell, whereas larger irregularly shaped elements occurred in the perinuclear region. These data suggest that lip31 had been transported out of the ER despite the fact that the lip31 carbohydrates had not been processed by Golgi enzymes (see above).

The subcellular distribution of lip31D9 was similar to that of lip31 (Fig. 3c). The lip31D16-expressing cells had immunoreactive molecules over their entire area but most of the antibody-staining was punctate (Fig. 3d). In contrast to lip31, the mutant molecules appeared to be mostly confined to vesicle-like structures of a relatively uniform size that were distributed throughout the cell, although some accumulation of larger, li-containing vesicles around the nucleus was observed. Staining of cells expressing lip31D29 demonstrated a uniform, diffuse fluorescence consistent with cell-surface expression. No obvious subcellular structures seemed to have accumulated this form of li (Fig. 3e).

These data suggest that lip31 contains a targeting signal that directs it to vesicular structures. Indeed, lip31 might have two such signals, because lip31D16 had a subcellular distribution that was different from that of lip31 or lip31D29. To identify the vesicular structures, we co-stained HeLa cells expressing lip31 and lip31D16, respectively, with fluorescein- and Texas-red-labelled antibodies against li, the transferrin receptor, the M6PR and LampA. Figure 3f and h show the distribution of lip31. The two types of lip31-containing vesicles were prominent in the perinuclear area. Many of the smaller vesicles contained both lip31 and M6PR, but most of the larger lip31-containing vesicles were only weakly stained by the M6PR antibodies (Fig. 3g). The LampA protein (Fig. 3i) was present in the large, vesicular structures together with lip31, but many of the smaller vesicles were devoid of this lysosomal marker. Transferrin receptor antibodies did not co-stain with lip31 (not shown).

lip31D16 occurred in vesicles that did not contain transferrin receptors and LampA. Some vesicles in the perinuclear area contained both lip31D16 and M6PR but most lip31D16-containing vesicles did not contain this late endosomal marker (not shown).

These data, and the biosynthetic experiments, suggest that lip33 never leaves the ER whereas lip31 and the truncated forms of li are transported out of the ER. Because the only difference between lip33 and lip31 is an N-terminal extension of 15 amino acids, this sequence could contain an ER retention motif. The presence of endosomal/lysosomal targeting signals in li was also apparent.

lip31 contains two distinct targeting signals

The difference in subcellular distribution between lip31, lip31D16 and lip31D29 suggested that more than one endosomal/lysosomal targeting signal might exist. To analyse the targeting signals in lip31 and lip31D16, we subcellularly fractionated cells transfected with either of the constructs. After biosynthetic labelling for 20 min and a chase of 2 h the cells were mixed with HeLa cells, whose early and/or recycling endosomes had accumulated radioactive transferrin and whose early and late endosomes had been labelled with horseradish peroxidase³⁰. The homogenized cells were gradient-centrifuged in 27% Percoll³¹⁻³². Figure 4a shows the distribution of endosomal and lysosomal markers. Immunoprecipitation of each fraction demonstrated that lip31 and lip31D16 were confined to the lighter fractions corresponding to pool C. Material in this

pool was further resolved by sedimentation in a 17% Percoll gradient³². Four fractions were analysed for content of the lysosomal marker beta-hexosaminidase (Fig. 4b), the early and late endosomal marker horseradish peroxidase (Fig. 4c), the early endosomal marker ¹²⁵I-labelled transferrin (Fig. 4d), lip31D16 (Fig. 4e) and lip31 (Fig. 4f). Class I molecules expressed on the cell surface were enriched in fraction 4.

The density distributions of lip31 and lip31D16 confirmed the immunofluorescence data and demonstrated the presence of these proteins in different subcellular compartments, which overlap with known endosomal markers only partially. As a large portion of lip31D16 became terminally glycosylated (see Fig. 2b), this form of li could occur in a post-Golgi compartment. That lip31D16 did not co-stain with transferrin receptors and that it was present in fractions of density lower than that of the

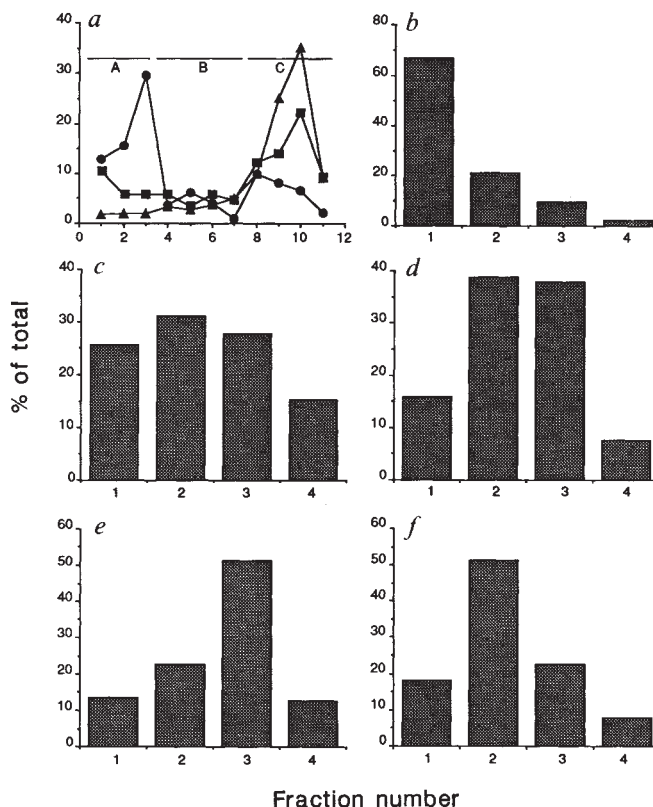


FIG. 4 lip31 and lip31D16 occur in separate subcellular compartments as distinguished by Percoll gradient centrifugation. Homogenized, lip31D16-transfected HeLa cells were separated on a 27% Percoll gradient (a) and individual fractions were assayed for content of the lysosomal marker β -hexosaminidase ($\bullet$), horseradish peroxidase (HRPO) ($\blacksquare$), a marker for late and early endosomes, and ¹²⁵I-labelled transferrin ($\blacktriangle$), a marker for early endosomes. Pool C, which contained more than 90% of lip31D16 (not shown), was separated on a 17% Percoll gradient and the amounts of β -hexosaminidase (b), HRPO (c), ¹²⁵I-labelled transferrin (d) and lip31D16 (e) were determined in each fraction. lip31-transfected cells were separately used in the same experiment and the distribution of lip31 is shown in f. The amounts of material in each fraction, as a percentage of the total material recovered, are shown on the ordinate.

METHODS. HeLa cells, transfected with lip31D16 or lip31, were separately mixed with HeLa cells whose endosomal subpopulations had been labelled with HRPO (8 mg ml⁻¹) for 1 h at 20 °C to uniformly label both early and late endosomes³⁰ or with ¹²⁵I-labelled diferric transferrin for 30 min at 37 °C to selectively label early and/or recycling endosomes³⁰. Cell surface ¹²⁵I-labelled transferrin was removed by sequential acid and neutral washes in the presence of the chelator desferrioxamine. The transfected HeLa cells, labelled with [³⁵S]methionine for 20 min followed by a 2-h chase, were combined with the endosome-labelled carrier cells before homogenization in 25 mM HEPES, 0.25 M sucrose, 1 mM EDTA, pH 7.4, and fractionation on sequential 12.5-ml Percoll gradients as previously described^{31,32}. li was immunoprecipitated after removal of the Percoll and quantitated by densitometry after SDS-PAGE and fluorography.

early endosomes containing ^{125}I -labelled transferrin, indicate that its subcellular localization may be a compartment with a different distribution of the classical markers for early and late endosomes. Given the complexity of the endosomal system³³, Iip31D16 might well occur in an endosomal subcompartment.

Iip31 accumulated in vesicles with a density similar to that for late endosomes. The apparent contradiction that Iip31 was virtually absent from fractions containing β -hexosaminidase and yet co-localized with LampA in the immunofluorescence stainings could be explained if it takes more than 2 hours for Iip31 to reach the lysosomal compartment. This notion is supported by the observation that only a small fraction of Iip31 was degraded during this time (see Fig. 2b).

The apparent lack of terminally glycosylated Iip31 molecules and their appearance in endosome/lysosome-like structures are at variance with the conventional transport route for endosomal/lysosomal proteins^{33–35}. Thus, Iip31 seems to be diverted from the default transport pathway before entering the Golgi complex. This novel pathway is blocked in chloroquine-treated cells, in which Iip31 becomes terminally glycosylated and changes its subcellular distribution (V.L., unpublished data). Although further experiments are needed to define precisely the transport pathway for Iip31, the present data raise the possibility that a direct route exists from the ER to the Iip31-containing endosome/lysosome-like vesicles, which may be a novel degradative pathway (see below).

Ii directs localization of HLA-DR α chains

We examined the distribution of class II HLA-DR α chains in cells simultaneously expressing Ii. Cells expressing only the DR α chain showed the reticulated, perinuclear staining pattern typical for proteins confined to the ER (Fig. 5a). The distribution of the DR α chain was not appreciably changed when cells expressed this chain with Iip33 (Fig. 5b). A different subcellular distribution of the DR α chain was observed in cells co-expressing Iip31; in this case DR α chains appeared mostly in the perinuclear region in large, vesicle-like structures. Small amounts of the chain were also present at the cell surface, as verified by FACS analyses (not shown), and in small vesicles enriched in the cell periphery (Fig. 5c). The staining pattern was indistinguishable from that for free Iip31 (see Fig. 3b). The DR α chain had a similar, if not identical distribution when it was co-expressed with Iip31D9 (not shown). Iip31D16 affected the distribution of the DR α chain—most of the antibody-reactive material occurred in small vesicles, mainly localized towards the periphery of the cell. A few large perinuclear structures and the cell surface were also stained (Fig. 5d). Also in this case the distribution of the DR α chain corresponded to that for Iip31D16 alone (see Fig. 3d). The simultaneous expression of DR α chains and Iip31D29 resulted in the staining of the cell surface only (Fig. 5e), as did Iip31D29 alone (see Fig. 2e). This suggests that neither Iip31D29 nor DR α chains contain signals that make them deviate from the default transport pathway.

To verify that the DR α chain was passively transported by Ii, we performed biosynthetic pulse-chase experiments. Whereas DR α chains alone and DR α chains with Iip33, Iip31 and Iip31D9 were completely susceptible to endo H throughout the chase, DR α chains with Iip31D16 and Iip31D29 became partially resistant to endo H (not shown). Consequently, the glycosylation pattern of the DR α chain faithfully reproduced the glycosylation patterns of the associated forms of Ii (see Fig. 2). This suggests that the DR α chain itself does not direct its subcellular localization and reinforces the notion that Iip31 is transported to late endosomes/lysosomes by a pathway not involving passage through the Golgi compartment.

Conclusions

Our results show that the N-terminal cytoplasmically exposed region of human Ii has three separate targeting signals. The

15-amino-acid residues that distinguish Iip33 from Iip31 seem to contain an ER retention motif similar to that in other ER-retained proteins^{27,36}. A consensus retention motif has been identified only for type I transmembrane proteins³⁷. Because Ii is a type II transmembrane protein, the critical residues in the putative Ii retention sequence cannot so far be identified. But the fact that the N-terminal region of Iip33 contains several basic residues conforms with the requirements for type I transmembrane protein-retention motifs³⁷.

The N-terminal region of Iip31 contains two other sorting signals. The most N-terminal signal operates in Iip31 and in the Iip31D9 mutant but it is disrupted in Iip31D16. Therefore, the seven-amino-acid residues that differ between Iip31D9 and Iip31D16 must contribute to this signal. Deletion of residues that differ between Iip31D16 and Iip31D29 seems to remove the third sorting signal. Although the existence of these two transport signals is obvious, it is less clear where they target Ii. The co-localization and subcellular fractionation data are consistent with Iip31 being *en route* to lysosomes by way of late endosomes, but as this pathway originates in the trans-Golgi network^{16,33}, one would expect Iip31 to become terminally glycosylated. Therefore, alternative routes have to be considered, including ones in which this M6PR-containing prelysosomal compartment is not part of the endocytic pathway. For example, because autophagosomes seem to originate from the ER and gradually obtain late endosomal marker proteins before fusing with lysosomes^{38,39}, Iip31 could be targeted to these structures. Further studies using endocytic tracers will help to distinguish between the possibilities.

The terminal glycosylation of Iip31D16 suggests that the third targeting signal operates only after Ii has passed the Golgi complex. This form of Ii does not co-localize with known markers for early endosomes, but it does co-localize with some recycling plasma membrane proteins in chloroquine-treated cells (V.L., unpublished data). This suggests that the third targeting signal may direct Ii to a subcompartment of the early endosomal system. Further studies are needed to clearly define this compartment.

The role of Ii is to bind to class II antigens. The targeting signals are, therefore, used primarily to guide class II molecules to the relevant subcellular compartments. Also, intracellular transport signals operate in a spatially, and therefore temporally, restricted manner. This is exemplified by Iip33 which has three targeting signal. But the ER retention signal obviously overrides the two endosomal signals.

The present data suggest that Ii controls the intracellular transport of class II molecules. Nascent class II α and β chains probably associate with Iip33 and Iip31 before assembly of class II molecules³. Those chains that are associated with Iip31 and fail to form heterodimers might be transported to autophagosomes for degradation. Iip31 might be equivalent to other proteins participating in the assembly of complex structures such as the T-cell receptor (see refs 40–42). We suggest that Ii is one of several proteins that guide unassembled subunits and misfolded protein from the ER to autophagosomes.

Individual class II chains retained in the ER by binding to Iip33 might be able to form heterodimers more quickly than those associated with Iip31. If so heterodimers bound to Iip33 could participate in reaction in which Iip33 is substituted for Iip31 to initiate transport out of the ER. Because the trimolecular complex is transported through the Golgi¹, the targeting signal that directs Iip31 to deviate from the default pathway has to be obscured by the association with the class II chains. It is reasonable to suggest that only the heterodimers have this capacity. This is consistent with our data demonstrating that the DR α chain alone does not affect the targeting of Iip31.

The second targeting signal present in Iip31 might become operative either in the trans-Golgi network or at the cell surface⁴³. The present data do not distinguish between these possibilities. If the first situation were to prevail, we would

conclude that sorting in the trans-Golgi network is incomplete because small amounts of Iip31 and the truncated Ii variants, alone or with DR α chains, occur at the cell surface. Alternatively, sorting in the trans-Golgi network to part of the endosomal compartment could precede transport to the cell

surface⁴⁴⁻⁴⁶. Although the exact transport route is unclear, it seems likely that the second targeting signal directs class II molecules to a compartment where they can divest themselves of Ii for peptide-binding to occur. □

Received 18 June; accepted 29 October 1990.

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ACKNOWLEDGEMENTS. Gifts of antibodies from S. Pfeffer and M. Williams are gratefully acknowledged. V.L. was the recipient of a Juvenile Diabetes Foundation fellowship and A.P. is supported by the Fonds National Suisse de la Recherche Scientifique. S.L.S. acknowledges a Lucille P. Markey Scholarship. This work was supported by the NIH (P.A.P. and V.Q.).

A yeast mitochondrial outer membrane protein essential for protein import and cell viability

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The gene encoding ISP42, an integral outer-membrane protein located at the yeast mitochondrial protein import site was cloned, sequenced and modified. Yeast cells depleted of ISP42 accumulate uncleaved mitochondrial precursor proteins and then die. ISP42 is the first mitochondrial membrane protein shown to be indispensable for protein import and cell viability.

MOST mitochondrial proteins are synthesized on cytoplasmic ribosomes and imported into mitochondria. Although several of the signals directing proteins to mitochondria have been characterized^{1,2}, little is known about the machinery that decodes these signals and transports proteins across the mitochondrial membranes. Several receptor-like proteins on the mitochondrial outer membrane have been described³⁻⁶, most of which are functionally redundant and interact with only a subset of precursors^{3,5} and none of which has been shown to be essential for mitochondrial protein import. Indeed, yeast cells lacking the receptor protein MAS70 are viable⁷ and still import all precursors tested into their mitochondria, although at lower rates than wild-type cells⁶.

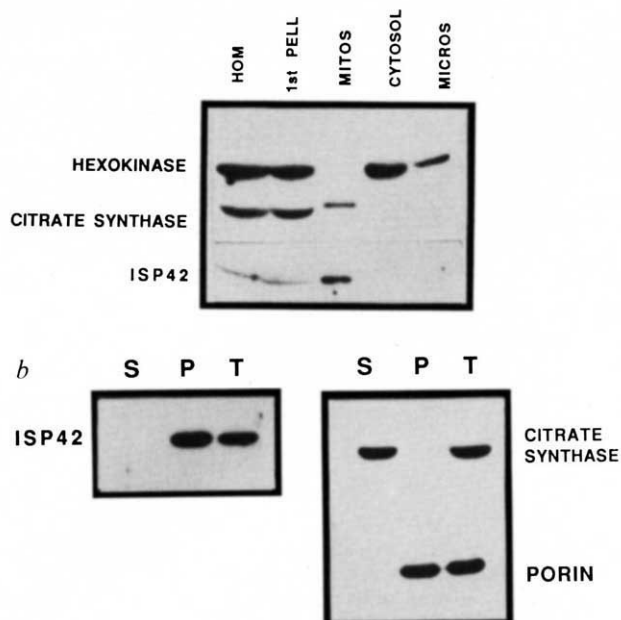
In a previous attempt to identify components of the yeast

mitochondrial protein translocation machinery, we photo-cross-linked a precursor protein that was 'stuck' in the import site to a mitochondrial outer membrane protein of relative molecular mass 42,000 (42K). We also demonstrated that IgGs monospecific for this protein inhibit protein import into isolated mitochondria⁸. We termed this protein import site protein 42 (ISP42) because it is at or near the site where isolated mitochondria import proteins. Our experiments, however, did not provide direct evidence that ISP42 participates in protein import. Here we report such evidence. We have now cloned and sequenced the ISP42 gene and show that depletion of ISP42 *in vivo* inhibits mitochondrial protein import and cell growth.

The ISP42 gene

By screening a library of random yeast genomic fragments in λ gt11 (ref. 9) with poly- and monoclonal antibodies against ISP42, we isolated a 0.78-kilobase (kb) *EcoRI* fragment that directs the synthesis of an ISP42-crossreacting β -galactosidase fusion protein. In northern analysis, this fragment hybridized to a single 1.2-1.3-kb RNA (not shown). To obtain the full-length ISP42 gene, we hybridized the 0.78-kb *EcoRI* insert to a library of large yeast genomic fragments in a yeast-*Escherichia coli* shuttle plasmid. Two of the cross-hybridizing recombinant plasmids isolated by this procedure cause overexpression of ISP42 in yeast (not shown). Restriction mapping of the two inserts, subcloning and sequence analysis (Fig. 1) revealed the presence of a single open reading frame capable of encoding a polypeptide of molecular mass 41,983. This value is in good agreement with that determined earlier⁸ by SDS-PAGE. Furthermore, this open

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When the haploid transformant is grown on galactose, it accumulates about 20 times more ISP42 than the wild-type parent strain. When this synthesis is repressed by shifting the transformant from galactose-containing to glucose-containing medium (Fig. 4a), the pre-existing ISP42 molecules are diluted by cell growth; wild-type levels of ISP42 are reached after 3–5 hours and sub-normal levels after 6 hours; after 15 hours, the level of ISP42 is at most 5% that of glucose-grown wild-type cells (Fig. 4b). At this point, the cells stop growing and eventually

die, even though the optical density of the culture still increases slightly.

When the level of ISP42 drops below the wild-type level, the cells accumulate uncleaved precursors of the F_1 -ATPase α and β subunits (Fig. 4b), citrate synthase and Mn-superoxide dismutase (not shown). Our earlier work has shown that cleavage of the two largest ATPase subunits is a valid measure of protein import provided the cells contain a normal level of the matrix-located processing protease¹⁰. The cells continue to accumulate

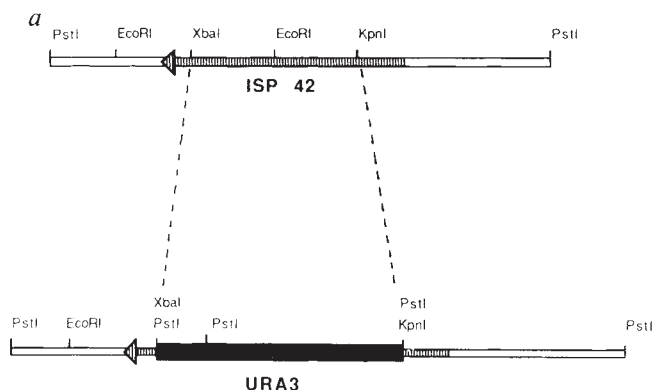
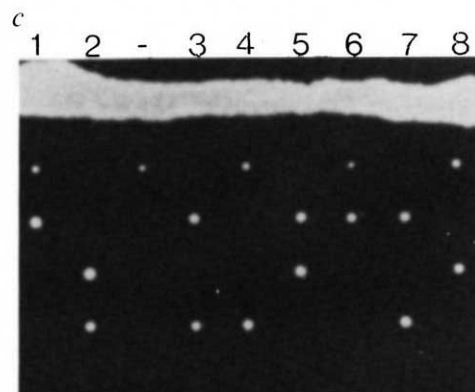
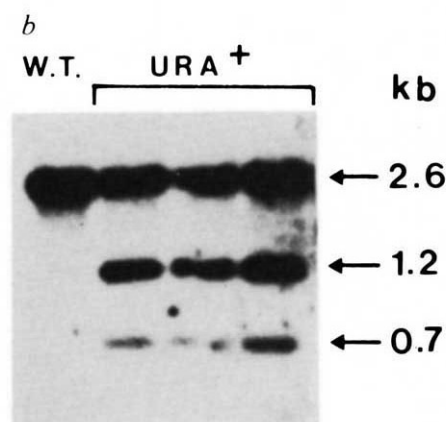


FIG. 3 ISP42 is essential for cell viability. *a*, One of the two copies of the ISP42 gene (hatched arrow) in the *ura3/ura3* diploid yeast strain YKB5 was disrupted³⁹ between its *XbaI* and *KpnI* sites by the yeast *URA3* gene (black box). *b*, If checked by Southern blotting⁴⁰, DNA from resulting uracil-sufficient diploids had a 2.6-kb *PstI* fragment derived from the uninterrupted gene, as well as the predicted 1.2- and 0.7-kb *PstI* fragments derived from the disrupted gene. *c*, Sporulation of such a heterozygous diploid (strain YKB6) and dissection of ascospores yielded two viable and two non-viable spores; all viable spores were uracil-requiring and thus carried the uninterrupted ISP42 gene. Numbers on top identify individual asci that were dissected. **METHODS.** The *URA3* gene was isolated as a *KpnI*-*XbaI* fragment from plasmid pUC13/18-*URA3* (M. Egerton, unpublished results) and ligated into plasmid pBR-ISP42 that had been digested with *XbaI* and *KpnI*. The *EcoRI*-*BglI* fragment from the resulting construct was used to transform strain YKB5 (*MATa/ura3, his4, leu2, Ade2/ade2, LYS2/lys2*) to uracil prototrophy. Chromosomal DNA⁴¹ from a uracil-prototrophic transformant was digested with *PstI*; the resulting fragments were separated by agarose gel electrophoresis, blotted to nitrocellulose³⁴ and examined for hybridization to the radioactive 2.6-kb *PstI* fragment derived from pBR-ISP42. WT, wild-type diploid.



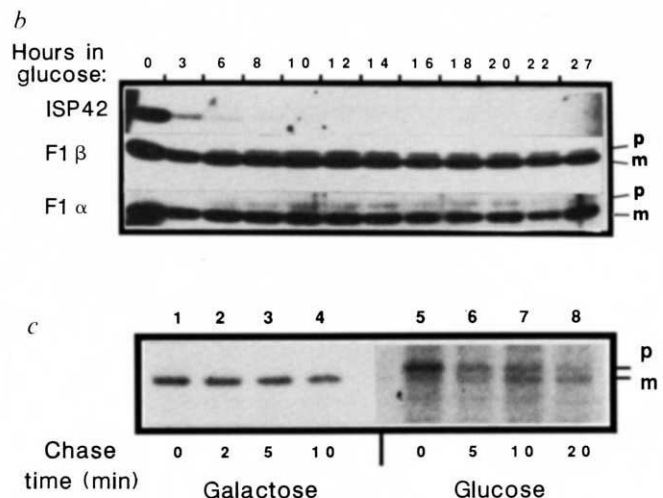
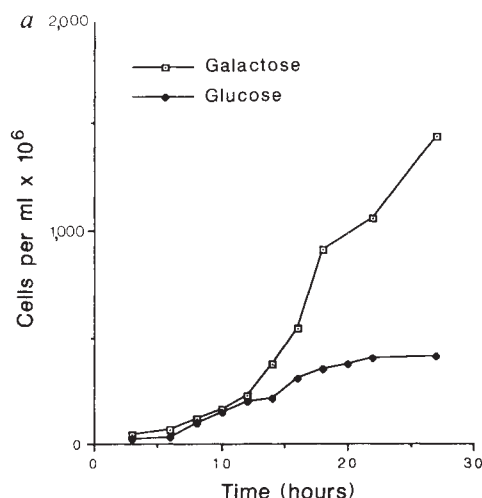


FIG. 4 Depletion of ISP42 causes growth arrest and accumulation of uncleaved mitochondrial precursor proteins. *a*, Strain YKB8 (*MATa*, *his4*, *ade2*, *ura3*, *leu2*, *isp42::LEU2*, *pGAL-ISP42*) was grown on YP-galactose medium to mid-logarithmic phase; the culture was diluted 50-fold into either fresh YP-galactose medium or into YP-glucose medium⁴². Cell growth after dilution was followed by measuring cell number or optical density at 600 nm (not shown). *b*, At the indicated times after dilution, total cell proteins were extracted¹⁰ and 150 μ g aliquots were analysed for ISP42 and the F_1 -ATPase α and β subunits by SDS-PAGE and immunoblotting³⁴. *c*, 14 h after dilution into galactose- or glucose-containing medium, maturation of the F_1 -ATPase β subunit in the cells was measured by a pulse-chase experiment⁶. p and m, Precursor and mature form.

METHODS. *a*, *pGAL-ISP42* was constructed by subcloning the *ISP42* gene into vector *pGAL*⁴³. A 10-mer *Bam*HI linker was introduced at the *Hin*fl site immediately 5' to the start ATG of the *ISP42* gene, and the gene was subcloned as a *Bam*HI, partial *Eco*RI fragment into *pGAL* that had been digested with *Bam*HI and *Eco*RI. The resulting plasmid was then used to construct strain YKB8 in which the plasmid supplies the only intact copy of

ISP42. To this end, one of the two *ISP42* genes in the diploid strain YKB5 was disrupted between its *Kpn*I and *Xba*I sites with the yeast *LEU2* gene to yield strain YKB7. After the disruption had been confirmed by Southern analysis (not shown), YKB7 was transformed with plasmid *pGAL-ISP42* and induced to sporulate. Ascospores were allowed to germinate on YP-galactose medium. Leucine and uracil prototrophs were identified and shown to be glucose-sensitive. Such a strain was designated YKB8. *c*, For pulse-chase, strain YKB8 was pregrown in galactose-containing semisynthetic medium⁴⁴, diluted into semi-synthetic medium containing either galactose (lanes 1–4) or glucose (lanes 5–8), allowed to grow for 14 h, and pulse-labelled⁶ with ³⁵S-methionine for 3 min at 30 °C. Labelling was stopped with cycloheximide and excess unlabelled methionine. Total proteins of the samples displayed in lanes 1 and 5 were then extracted¹⁰; samples shown in lanes 2–4 and 6–8 were first chased for the indicated times at 30 °C before being extracted. Extracted proteins were analysed by immunoprecipitation with rabbit anti-serum against the F_1 -ATPase β subunit followed by SDS-PAGE and fluorography.

some processed F_1 -ATPase subunits even after 15 hours of growth on glucose; this may reflect the fact that synthesis of these subunits is strongly repressed by glucose¹¹ and that the residual *ISP42* molecules may still be able to cope with slowly produced precursor molecules.

The inhibition of protein import into mitochondria of *ISP42*-depleted cells can be confirmed by pulse-chase experiments. Cells grown in galactose completely process the F_1 -ATPase α - and β -subunits within 3 min; 14 hours after inhibition of *ISP42* synthesis, however, the half-lives of these precursors *in vivo* are increased to between 10 and 20 min; (Fig. 4c). Thus, *ISP42*-depleted cells import these precursors five- to tenfold more slowly than *ISP42*-sufficient cells.

To demonstrate that the precursors accumulated in *ISP42*-depleted cells results from inhibition of import rather than of processing, we performed subcellular fractionation of these cells to determine the localization of the accumulated precursors (not shown). Precursors are not detected in any of the subcellular fractions, indicating that they are rapidly degraded on homogenization; this phenomenon has previously been shown for the F_1 -ATPase β subunit precursor accumulated by depletion of cytosolic heat-shock proteins¹². To explore the localization of the accumulated precursors further, we asked whether the slow chase found in *ISP42*-depleted cells is sensitive to the uncoupler carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP), which blocks one of the earliest steps in mitochondrial protein import¹³. We found that in such cells the chase is indeed blocked by CCCP, indicating that the accumulated precursor had not yet been transported beyond the potential-requiring step (Fig. 5). In the absence of the CCCP, 38% of the pulse-labelled precursor is converted to mature form whereas in its presence only 2% is converted. We conclude that the precursors

accumulating in *ISP42*-depleted cells have not been transported across the mitochondrial membranes.

Depletion of *ISP42* does not affect protein translocation across the endoplasmic reticulum: processing of the *GAS1* gene product (which is transported to the plasma membrane, ref. 14) or of carboxypeptidase Y (which is transported to the vacuole, refs 15, 16) are unaffected (not shown). The inhibition of mitochondrial protein import by *ISP42* depletion thus appears to be specific. This is also supported by our finding (not shown) that

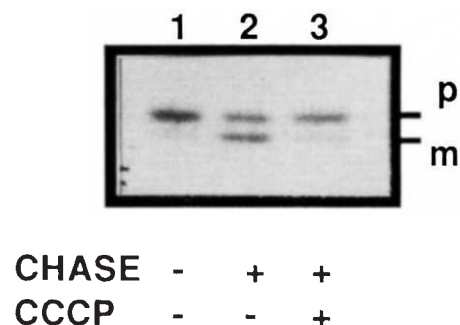


FIG. 5 A mitochondrial precursor protein accumulated by *ISP42*-depleted cells has not been properly imported into the mitochondria. The slow chase of the precursors to the F_1 -ATPase β -subunit to the mature form is sensitive to the uncoupler carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) which blocks one of the earliest import steps. Pulse-labelling and pulse-chase in the presence of CCCP were carried out on cells grown in glucose for 12 h; the pulse was for 3 min, as described in Fig. 4c, and the chase was in the presence or absence of 40 μ M CCCP for 15 min¹⁰. Extracted proteins were analysed as described in Fig. 4c. Lane 1, pulse; lane 2, chase; lane 3, chase in the presence of CCCP.

wild-type cells shifted from galactose- to glucose-containing medium do not accumulate mitochondrial precursor proteins.

When yeast cells are depleted of calmodulin, they die without accumulating mitochondrial precursor proteins¹². Thus, cell death by itself does not cause accumulation of mitochondrial precursor proteins in yeast. To verify this for the yeast strain used here, we placed its single plasma-membrane ATPase gene^{17,18} under control of a galactose-inducible and glucose-repressible promoter. Because plasma membrane ATPase is an essential protein for yeast¹⁸, the cells stop growing after approximately 15 hours in glucose, yet do not accumulate uncleaved mitochondrial precursor proteins (not shown). We conclude that depletion of ISP42 from yeast cells specifically interferes with mitochondrial protein import because ISP42 is a component of the import machinery.

Conclusions

We have shown here that ISP42 is essential for cell viability and for import of precursor proteins into mitochondria. The other known members of this family of mitochondrial proteins are the two subunits of the matrix-processing protease (encoded by *MAS1* and *MAS2*; refs 19–21); mitochondrial hsp60 (encoded by *MIF4*; ref. 22); and mitochondrial hsp70 (encoded by *SSC1*; refs 23, 24). ISP42 is the only membrane protein of this family. Several other outer-membrane proteins^{7,25,26}, including a mitochondrial import receptor^{6,7}, can be deleted from yeast cells without serious effects on cell growth. Unlike some of the functionally redundant mitochondrial import receptors, ISP42 thus appears to catalyse a central step of protein import. As it is in close contact with a partially translocated precursor protein⁸ and is resistant to digestion by externally added proteases⁶, ISP42 may be a subunit of a proteinaceous import channel through the outer membrane (see below).

Although ISP42 behaves like an integral membrane protein, its amino-acid sequence is not that of a typical membrane-

embedded protein²⁷. There is a similar apparent discrepancy with several pore-forming proteins that span membranes in non-helical conformations and generate structures with hydrophilic transmembrane channels²⁸. Several regions of the ISP42 protein sequence are amphipathic and could contribute to the formation of a hydrophilic channel. Such a topology would certainly fit with the transmembranous location of the stuck intermediate which crosslinks to ISP42. The channel could be an ISP42 homo-oligomer or could contain other proteins. We favour the second possibility as overproduction of ISP42 causes aberrant intramitochondrial sorting of the protein (not shown). Binding to another protein could also explain how ISP42, which lacks a typical N-terminal mitochondrial targeting signal, inserts into the outer membrane. We are searching for possible candidates for this protein.

Translocation of proteins into the mitochondrial matrix requires the sequential action of two distinct translocation systems, one associated with the outer mitochondrial membrane and the other with the inner membrane²⁹. ISP42 is clearly part of the outer membrane system. As it is not uniquely found in sites of contact between the two mitochondrial membranes, contact sites functioning in protein import may form only in response to a mitochondrial precursor which has bound to one of the import receptors on the mitochondrial outer membrane.

We have just learned that Kiebler *et al.*³⁰ (accompanying paper) have recently cloned and sequenced the gene encoding a 38K outer membrane protein (MOM38) from *Neurospora crassa* mitochondria (which appears to be part of the protein import machinery). The sequence of MOM38 is significantly identical to that of ISP42, indicating that MOM38 is the *Neurospora* homologue of ISP42. Involvement of MOM38 in protein import was initially deduced from its association with other import receptors. It thus seems that ISP42 and MOM38, which were identified by entirely different approaches, may perform the same function in the two organisms. □

Received 13 September; accepted 9 November 1990.

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ACKNOWLEDGEMENTS. Supported by grants from the Swiss National Science Foundation, the US Public Health Service and the Human Frontier Science Program Organization (to G.S.), and by a fellowship from EMBO (to D.V.). We thank Eric Kuebler for assistance with ascospore dissection, Patrick Linder for plasmid pGAL, Ramon Serrano for a plasmid-borne copy of the manipulated PMA1 gene, Prof. Hans Trachsel for the λ gt11 library and Abraham Amsterdam for help with immunoelectron microscopy.

Identification of a mitochondrial receptor complex required for recognition and membrane insertion of precursor proteins

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The mitochondrial import receptors MOM19 and MOM72 form a complex with two other proteins of the mitochondrial outer membrane, MOM38 and MOM22. This receptor complex is involved in recognition, membrane insertion and translocation of precursor proteins with MOM38 constituting (at least part of) the general insertion site GIP.

TRANSLOCATION of precursor proteins into and across organelle membranes is an essential step in the biogenesis of a number of cell organelles, such as the endoplasmic reticulum, mitochondria, chloroplasts and peroxisomes¹. Intriguing questions are how the cytosolically synthesized precursor proteins are targeted to the correct organelle and how the precursor polypeptides are transferred across the membrane barriers.

By analysing translocation intermediates, the import of precursor proteins into mitochondria has been dissected into a series of steps²⁻⁴, including the specific binding of precursors to receptors on the mitochondrial surface, the insertion of precursors into the outer mitochondrial membrane at a general insertion site, GIP, and the further translocation of precursors through contact sites between both mitochondrial membranes. Several of the components mediating the transfer of precursor proteins have been identified. Cytosolic hsp70 heat-shock proteins are involved in preventing misfolding of the polypeptide chains⁵⁻⁸. The import of most precursor proteins occurs by way of the general import receptor MOM19, a mitochondrial outer membrane protein of relative molecular mass 19,000 (M_r 19K) in *Neurospora crassa*^{9,10}. MOM72, a second surface receptor identified in *N. crassa* and in yeast mitochondria, is important for the import of ADP/ATP carrier, the most abundant mitochondrial membrane protein^{10,11}. A 42K protein of the yeast mitochondrial outer membrane seems to interact with precursor proteins, although its exact function is unknown¹². Moreover, several proteins of the mitochondrial matrix have been identified as components of the protein import machinery, including the heat-shock proteins mitochondrial hsp70 (ref. 13) and hsp60 (refs 14, 15), the mitochondrial processing peptidase MPP and the processing enhancing protein PEP^{16,17}.

Little is known about the components mediating insertion and membrane translocation of mitochondrial precursor proteins. Both receptors, MOM19 and MOM72, seem to transfer the precursor proteins to a common insertion site in the outer membrane, termed the general insertion protein (GIP)^{18,19}. An isolated mitochondrion contains roughly 200–400 GIP sites¹⁸ (for comparison, one mitochondrion contains a similar number of receptor sites, yet roughly 10 times the number of translocation contact sites^{9,11,20,21}). How are the precursors transferred from the receptors to GIP and what is the structural equivalent

of GIP? In this report, we identify a high-molecular-weight complex in the mitochondrial outer member that contains both receptors and two additional components. One of them, MOM38, apparently represents GIP or at least a part of it. Thus, the receptors directly interact with GIP and thereby donate the precursors to this membrane insertion site. Furthermore, we present evidence that this 'receptor complex' is also involved in the translocation of precursor proteins through mitochondrial contact sites.

Identification of the receptor complex

To investigate possible interactions of the mitochondrial import receptors with other outer membrane proteins, isolated *N. crassa* mitochondria were lysed with the mild detergent digitonin and analysed by gel filtration. MOM19 fractionated in a range expected for molecules of about 400–600 K (Fig. 1a). A considerable amount of MOM72 also appeared in those fractions. Although an exact sizing is evidently impossible as a result of the presence of detergent, this finding implied that the receptors may not be present as monomers, but rather in oligomeric forms.

To purify putative protein complexes, we made use of specific antibodies directed against MOM19 (ref. 9). *N. crassa* cells were grown in the presence of [³⁵S]sulphate and isolated mitochondria lysed with digitonin. Immunoprecipitation was then performed with anti-MOM19 antibodies prebound to protein A-Sepharose and the precipitates were analysed by SDS-polyacrylamide gel electrophoresis and fluorography. Three protein bands of apparent M_r s 22K, 38K and 72K were coprecipitated with MOM19 (Fig. 1b, lane 1). The 38K protein and part of the 72K and 22K proteins also cofractionated with MOM19 on gel filtration (Fig. 1a). Controls show the specificity of the coprecipitation. None of the four components was precipitated with pre-immune serum obtained from the rabbit before immunization with MOM19 (Fig. 1b, lane 3). After degradation of MOM19 by pretreating intact mitochondria with low concentrations of trypsin (leading to degradation of a few mitochondrial surface proteins^{9,18,22}), none of the proteins was coprecipitated (Fig. 1b, lane 2). As MOM38 is not degraded by this treatment with trypsin (see Fig. 6a), coprecipitation of MOM38 apparently results from its association with a mitochondrial surface component and not from unspecific precipitation. Lanes 9–12 of Fig. 1b show the total amount of mitochondrial proteins (for each lane, one tenth of the amount of mitochondria used for one immunoprecipitation was taken), demonstrating that the immunoprecipitated proteins represent a selective and very minor fraction of mitochondrial proteins, in agreement with the low abundance of these four outer membrane proteins (see below). In view of the large number of mitochondrial proteins that are 10- to 100-fold more abundant, an unspecific coprecipitation of these four outer membrane proteins is extremely unlikely. In the presence of Triton X-100 and 300 mM NaCl (standard conditions for immunoprecipitation) only MOM19 was brought down by anti-MOM19 antibodies (Fig. 1b, lane 5). Similarly, after dissolving mitochondria in SDS-containing

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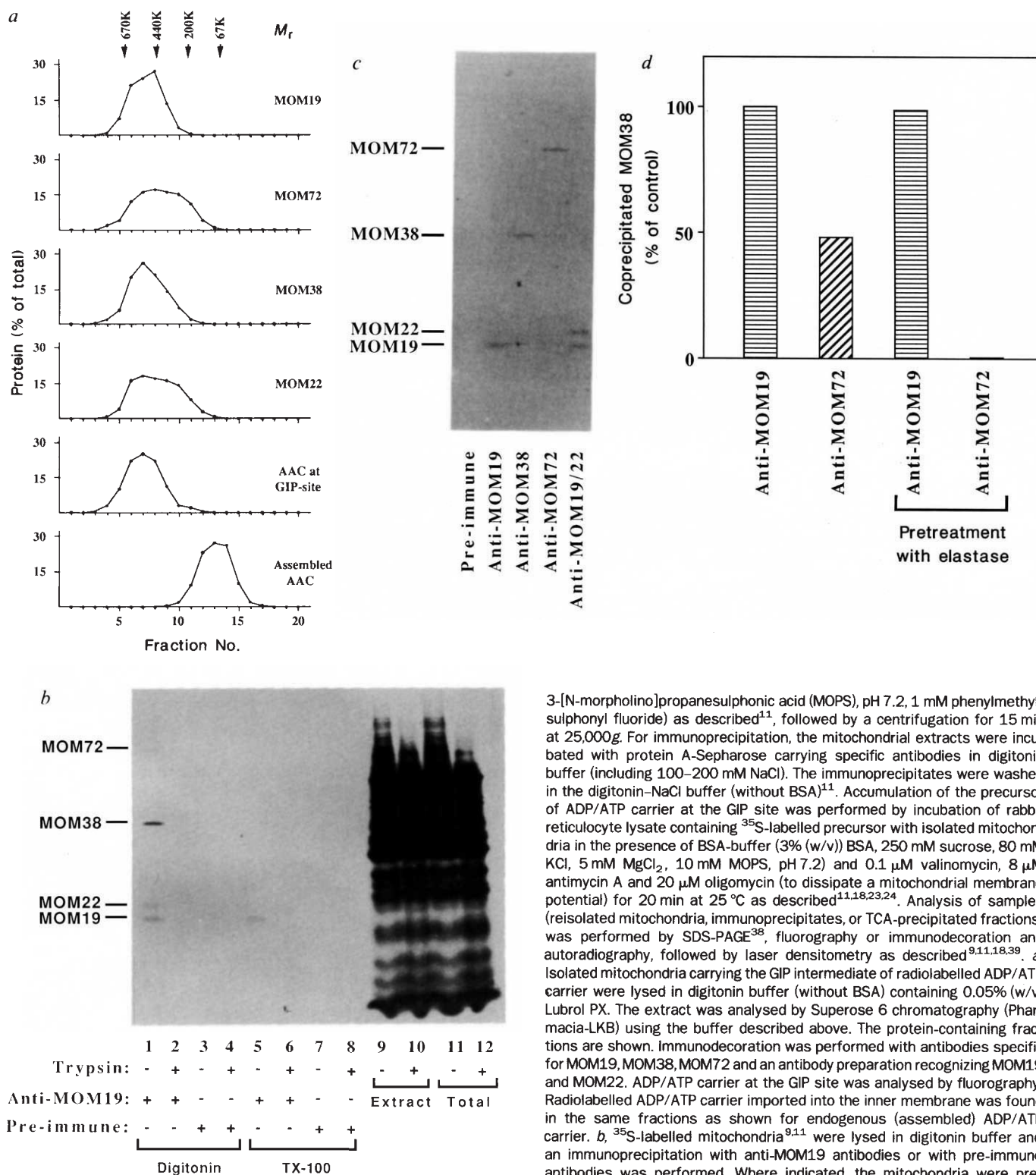
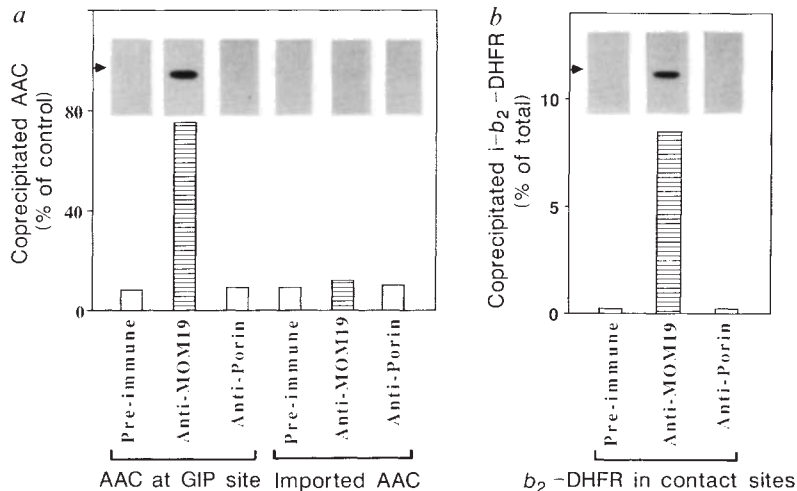


FIG. 1 Identification of a receptor complex in the outer membrane of *N. crassa* mitochondria. **a**, Analysis of outer membrane proteins and ADP/ATP carrier by gel filtration. AAC, ADP/ATP carrier; assembled AAC, mature dimeric ADP/ATP carrier in the inner membrane. Arrows indicate M_r standards. **b**, Precipitation of a protein complex with anti-MOM19 antibodies. Trypsin, mitochondria pretreated with trypsin; TX-100, Triton X-100; total, labelled mitochondrial proteins; extract, mitochondrial proteins extracted with digitonin. **c**, Identification of mitochondrial outer membrane proteins in the protein complex. **d**, Coprecipitation of MOM38 with anti-MOM72 antibodies. Elastase, mitochondria pretreated with elastase. **METHODS.** Mitochondria ($1 \text{ mg protein ml}^{-1}$) were lysed in digitonin buffer (0.5% (w/v) digitonin, 3% (w/v) BSA, 250 mM sucrose, 1 mM EDTA, 10 mM

3-[N-morpholino]propanesulphonic acid (MOPS), pH 7.2, 1 mM phenylmethylsulphonyl fluoride) as described¹¹, followed by a centrifugation for 15 min at 25,000g. For immunoprecipitation, the mitochondrial extracts were incubated with protein A-Sepharose carrying specific antibodies in digitonin buffer (including 100–200 mM NaCl). The immunoprecipitates were washed in the digitonin–NaCl buffer (without BSA)¹¹. Accumulation of the precursor of ADP/ATP carrier at the GIP site was performed by incubation of rabbit reticulocyte lysate containing ³⁵S-labelled precursor with isolated mitochondria in the presence of BSA-buffer (3% (w/v)) BSA, 250 mM sucrose, 80 mM KCl, 5 mM MgCl₂, 10 mM MOPS, pH 7.2) and 0.1 μM valinomycin, 8 μM antimycin A and 20 μM oligomycin (to dissipate a mitochondrial membrane potential) for 20 min at 25 °C as described^{11,18,23,24}. Analysis of samples (reisolated mitochondria, immunoprecipitates, or TCA-precipitated fractions) was performed by SDS-PAGE³⁸, fluorography or immunodecoration and autoradiography, followed by laser densitometry as described^{9,11,18,39}. **a**, Isolated mitochondria carrying the GIP intermediate of radiolabelled ADP/ATP carrier were lysed in digitonin buffer (without BSA) containing 0.05% (w/v) Lubrol PX. The extract was analysed by Superose 6 chromatography (Pharmacia-LKB) using the buffer described above. The protein-containing fractions are shown. Immunodecoration was performed with antibodies specific for MOM19, MOM38, MOM72 and an antibody preparation recognizing MOM19 and MOM22. ADP/ATP carrier at the GIP site was analysed by fluorography. Radiolabelled ADP/ATP carrier imported into the inner membrane was found in the same fractions as shown for endogenous (assembled) ADP/ATP carrier. **b**, ³⁵S-labelled mitochondria^{9,11} were lysed in digitonin buffer and an immunoprecipitation with anti-MOM19 antibodies or with pre-immune antibodies was performed. Where indicated, the mitochondria were pretreated with trypsin as described^{18,23}. For lanes 5–8, the immunoprecipitates were washed in a buffer containing 1% (w/v) Triton X-100 and 300 mM NaCl as described⁴⁰. Lanes 1–8 are derived from samples containing 50 μg mitochondrial protein, lanes 9–12 correspond to 5 μg mitochondrial protein. **c**, The protein complex was precipitated with anti-MOM19 antibodies as described for (b) and dissolved in SDS-containing sample buffer. After a 40-fold dilution with buffer containing Triton X-100, a second immunoprecipitation with the indicated antibodies was performed under standard conditions⁴⁰. **d**, The protein complex was precipitated with anti-MOM19 or anti-MOM72 antibodies, and MOM38 was analysed as described above. Where indicated, the mitochondria were pretreated with elastase ($1 \mu\text{g ml}^{-1}$) as described^{10,18}. The amount of MOM38 precipitated with anti-MOM19 antibodies was set to 100%.

FIG. 2 Copurification of translocation intermediates with the receptor complex. *a*, Copurification of the GIP intermediate of ADP/ATP carrier. The arrowhead indicates copurified ADP/ATP carrier (AAC). *b*, Copurification of the contact site intermediate of b_2 -DHFR. The arrowhead indicates copurified intermediate-sized i - b_2 -DHFR.

METHODS. *a*, ADP/ATP carrier precursor was accumulated at the GIP site of isolated mitochondria as described in the legend to Fig. 1. For import of ADP/ATP carrier into the inner membrane, 8 mM potassium ascorbate and 0.2 mM N,N,N',N'-tetramethylphenylenediamine were added (to generate a mitochondrial membrane potential) and valinomycin, antimycin and oligomycin were omitted²³. The reisolated mitochondria were lysed in digitonin buffer and immunoprecipitations were performed as described in the legend to Fig. 1. The amount of ADP/ATP carrier at the GIP site or of imported ADP/ATP carrier (determined by immunoprecipitation with anti-AAC antibodies²³) was set to 100%. *b*, Reticulocyte lysate containing ³⁵S-labelled precursor of cytochrome b_1 (1-167)-DHFR²¹ was preincubated with 1 μ M methotrexate for 5 min at 0 °C and then added to isolated mitochondria in the presence of a membrane potential as described above²¹. Lysis of the reisolated mitochondria with digitonin buffer and further treatment were



performed as described above. The total amount of i - b_2 -DHFR was set to 100%.

buffer, only MOM19 was precipitated, emphasizing the monospecificity of the anti-MOM19 antibodies⁹.

The coprecipitated components were identified using a collection of antisera prepared against mitochondrial outer membrane proteins from *N. crassa*^{9,11}. We found that the 72K protein was indeed MOM72 (Fig. 1c). The 38K protein was recognized by antibodies prepared against an outer membrane protein of this size and consequently termed MOM38. Finally, the 22K protein was immunoprecipitated by an antiserum that recognizes both

MOM19 and a 22K outer membrane protein, now termed MOM22 (Fig. 1c). Each of the four components of the complex represents roughly 0.01–0.04% of total mitochondrial protein as assessed by the abundance of the Coomassie blue-stained protein bands.

The molar ratios of MOM19:MOM22:MOM38:MOM72 after coprecipitation with anti-MOM19 antibodies are about 1:0.6 (± 0.2):1.0 (± 0.1):0.5 (± 0.15), determined as the average of 10 experiments. Assuming that the receptors MOM19 and

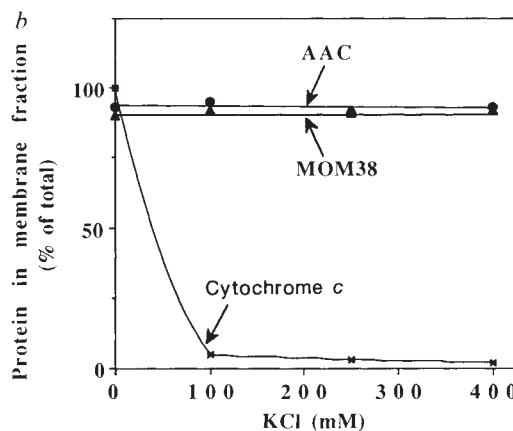
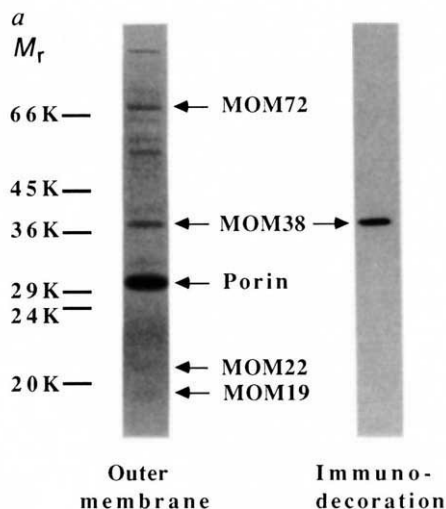
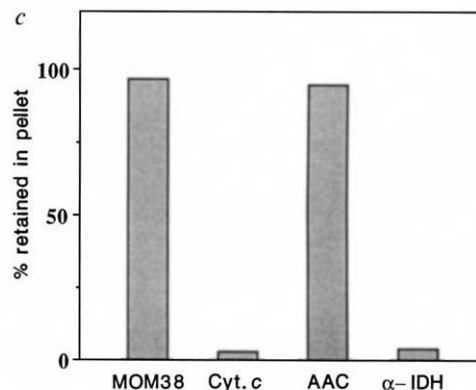


FIG. 3 MOM38 is an integral protein of the mitochondrial outer membrane. *a*, Protein pattern of the outer membrane of *N. crassa* mitochondria and immunodecoration of MOM38. *b*, MOM38 is not released from the membranes by salt and sonication. *c*, MOM38 is membrane-associated at pH 11.5. Cyt. *c*, cytochrome *c* (intermembrane space); AAC, ADP/ATP carrier (inner membrane); α -IDH, α subunit of isocitrate dehydrogenase (matrix).

METHODS. *a*, Mitochondrial outer membranes of *N. crassa* were isolated as described⁹ and resolved by SDS-PAGE. Proteins were stained with Coomassie blue R-250. Mitochondrial proteins were transferred to nitrocellulose and immunodecorated with anti-MOM38 antibodies^{9,11}. A similar result was obtained when purified outer membrane proteins were immunodecorated with anti-MOM38 antibodies. *b*, Mitochondria (0.2 mg protein ml^{-1}) were sonicated at various concentrations of KCl^{9,11}. Membranes and supernatants were separated by centrifugation for 60 min at 166,000g. *c*, Mitochondria (0.1 mg protein ml^{-1}) were incubated in 100 mM Na_2CO_3 (pH 11.5) for 30 min at 0 °C. Separation of pellets and supernatants was performed as described⁴¹.



MOM72 are present in roughly equimolar amounts in mitochondria^{9,11}, this implies that only about 50% of the MOM72 molecules are coprecipitated with anti-MOM19 antibodies (a similar conclusion applies to MOM22). The extent of coprecipitation of MOM38 with anti-MOM19 antibodies compared with that with anti-MOM72 antibodies is shown in Fig. 1d (we previously showed that the anti-MOM19 antibodies quantitatively precipitated MOM19 and similarly the anti-MOM72 antibodies quantitatively precipitated MOM72 (refs 9, 11)). About half of the MOM38 molecules that were coprecipitated with anti-MOM19 antibodies were coprecipitated with anti-MOM72 antibodies, suggesting that (at least) two types of MOM19/MOM38 complexes exist: those containing MOM72 and those not containing MOM72. The finding that only a fraction of the MOM72 molecules is associated with the MOM19/MOM38 complex agrees well with the previous observation of a partially different distribution of MOM19 and MOM72 over the outer membrane. In particular, MOM72 is more enriched in contact sites areas than MOM19 (refs 9, 11).

MOM72 is selectively degraded by treatment of mitochondria with low concentrations of elastase^{10,11}, whereas other outer membrane proteins, including the three other components of the complex, are not affected. After such pretreatment, anti-MOM72 antibodies did not coprecipitate the other complex components as shown for MOM38 in Fig. 1d. This represents an additional independent control for the specificity of the coprecipitation.

Functions of the receptor complex

Does this mitochondrial receptor complex have further functions in addition to being involved in recognition of precursors? Mitochondrial precursor proteins can be arrested at different stages of their translocation pathway (translocation intermediates), including as intermediates inserted into the outer membrane at the GIP site^{18,23,24} and intermediates spanning both mitochondrial membranes at contact sites^{20,21,25-27}. We asked whether precursors trapped at these intermediate stages are associated with the receptor complex: that is, can they be copurified with the complex.

The GIP intermediate of the precursor of ADP/ATP carrier was formed by incubating precursor synthesized *in vitro* with isolated mitochondria in the presence of ATP, but in the absence of an electrical potential across the inner membrane^{18,23,24}. Mitochondria were then lysed with digitonin and immunoprecipitation performed with anti-MOM19 antibodies. Figure 2a shows that the GIP intermediate of the ADP/ATP carrier was efficiently coprecipitated by anti-MOM19 antibodies, whereas antibodies from pre-immune serum or antibodies directed against the major outer membrane protein porin did not precipitate the precursor. In contrast, we previously reported that ADP/ATP carrier bound to its high-affinity receptor MOM72 on the mitochondrial surface was virtually not coprecipitated with anti-MOM19 antibodies¹¹, indicating that at this stage the precursor is not associated with the complex. As expected, both intermediates of the ADP/ATP carrier, the surface-bound form and the GIP-associated form, were coprecipitated with antibodies to MOM72 (ref. 11 and data not shown). On the other hand, ADP/ATP carrier fully imported into the inner membrane was neither coprecipitated with anti-MOM19 nor with anti-MOM72 antibodies (Fig. 2a, and ref. 11). ADP/ATP carrier at the GIP site, but not fully imported dimeric ADP/ATP carrier, also cofractionated with MOM19 in a gel filtration (Fig. 1a).

Together with our previous findings that the precursor of ADP/ATP carrier first binds to its receptor MOM72, then inserts into the GIP site and eventually is translocated into the inner membrane^{11,18,23,24}, these results suggest the following import pathway for the ADP/ATP carrier. The precursor predominantly binds to the MOM72 molecules that are not present in the complex. On assembly of MOM72 into the complex, the precursor

is delivered to the general insertion site, GIP, that seems to be formed by one or more components of the receptor complex (as discussed below, MOM72 itself does not constitute the GIP site). From the GIP site, the precursor is then transported into the inner membrane and assembled into the dimeric form²⁸.

In order to arrest a precursor stably in mitochondrial contact sites, a fusion protein between the 167 N-terminal amino-acid residues of the precursor of cytochrome *b*₂ and entire dihydrofolate reductase (*b*₂-DHFR) was preincubated with methotrexate²¹. The tertiary structure of the DHFR-domain was thereby stabilized and, on addition to isolated mitochondria in the presence of ATP and a membrane potential, the cytochrome *b*₂-part of the fusion protein was inserted into the mitochondrial membranes and the presequence cleaved off in the matrix. The folded DHFR remained on the cytosolic side and the precursor therefore spanned both mitochondrial membranes²¹. The mitochondria were lysed with digitonin and a co-precipitation with anti-MOM19 antibodies was performed. About 8–10% of the contact site intermediates were coprecipitated, whereas control antibodies did not show an effect (Fig. 2b). The efficiency of coprecipitation agrees with the relative abundances of translocation sites and receptor complexes (about 10-fold more

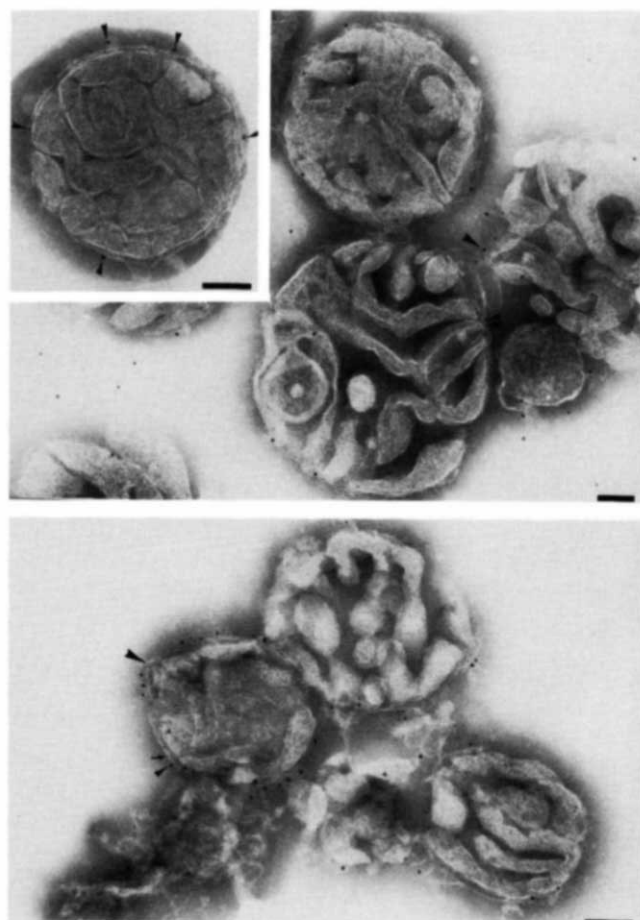


FIG. 4 Immunocytochemical localization of MOM38 in the mitochondrial outer membrane. Upper half, cryosections of mitochondria labelled with anti-MOM38 antibodies and protein A-gold particles (large arrowhead); small arrowheads, outer membrane. Insert, cryosections of mitochondria labelled with anti-MOM38 antibodies and protein A-gold particles (arrowheads). Lower half, mitochondria pre-labelled with anti-porin antibodies and 9-nm protein A-gold particles (small arrowhead) were cryosectioned and labelled with anti-MOM38 antibodies and 6-nm protein A-gold particles (arrow); large arrowhead, outer membrane. Bars represent 100 nm.

METHODS. Cryosections of *N. crassa* mitochondria were prepared and labelled as described^{9,11,42,43}.

translocation sites). Moreover, MOM38 can be crosslinked to a precursor protein arrested in contact sites (J. Rassow, M. Wiedmann, N. P. and W. N., unpublished results). It is concluded that the receptor complex is in contact with a fraction of the contact site intermediates, suggesting that the complex is also involved in the translocation of precursors through contact sites. Because of its low abundance compared with translocation contact sites, the receptor complex is probably not a permanent structural part of such sites. We propose that the receptor complex may transiently interact with contact sites and partake in the membrane insertion and translocation of precursor proteins, while (more abundant) proteins that have not been identified so far would form the structural basis of the contact sites.

MOM38 and the GIP

We analysed the properties of MOM38, the major complex component besides MOM19, in order to determine its possible function. Figure 3a shows the protein band corresponding to MOM38 on a Coomassie blue-stained SDS-polyacrylamide gel of purified outer membranes. MOM38 is not released from the membranes by sonication of mitochondria at various salt concentrations (Fig. 3b) or by treatment at alkaline pH (Fig. 3c), indicating that MOM38 is an integral membrane protein. Its localization in the outer mitochondrial membrane was confirmed by immunocytochemistry. Cryosections of *N. crassa* mitochondria were labelled with antibodies directed against MOM38

followed by protein A-gold particles. Figure 4 (upper part including insert) shows that the vast majority of gold particles were close to the outer membrane. As a further control for the outer membrane localization of MOM38, its colocalization with porin is shown (Fig. 4, lower part).

A full-length complementary DNA clone of MOM38 was isolated from a λ gt11 library of *N. crassa* cDNA²⁹. The nucleotide sequence and the deduced amino-acid sequence are shown in Fig. 5a. A protein with 349 amino-acid residues and M_r 38,108, containing at least one possible membrane spanning sequence, is predicted. Two stretches with similarity to the A and B regions, respectively, of an ATP-binding consensus sequence³⁰ are present in the C-terminal half of MOM38 (Fig. 5a). This finding may be related to the previously observed dependence on ATP for the transfer of precursor proteins from the receptor sites to the GIP site²⁴. Two regions with a predicted α -helical conformation (amino acid residues 142–154 and 329–340) are characterized by a high content of negatively charged amino-acid residues and the complete absence of positively charged residues. In both cases, the negative charges are located on one side of the helix. These regions are thus putative candidates for sequences interacting with the positively charged mitochondrial presequences^{4,31,32}. It is also notable that MOM38 contains an unusually high proportion of phenylalanine residues (7% of all residues).

MOM38 contains a sequence of about 70 amino-acid residues

a

28 GTCCCTGTCTACTGCGACGGTTACAATC

+ 1 ATGGCTTCGTTTTCACCGAGTGCGCTTTGGGATGCTGGCGACAAATGCCAATTATTCG

1 M A S F S T E S P L A M L R D N A I Y S

+ 61 AGCGTTTCGATGCGTTCAAGCGCTTTCAAGAAAGGAGAAACAGTTTCGCTTTTCAAC

21 S L S D A F N A F Q E R R K Q F G L S N

+ 121 CCGGCGAGATCGAGACCATGCGCCGCGAGGTCCAAAGCGATACCTCTCAACCACTAC

41 P G T I E T I A R E V Q R D T L L T N Y

+ 181 ATGTTCTCTGGCTCCGCGCGACGTCACCAAGGCTTTACGCTCGCCCTCTCTTCQAA

61 M F S G L R A D V T K A F S L A P L F Q

+ 241 GTTCCACACGTTTTCGATGGGCGAGAGGTGAAAGCTTATGCTTTGCTGCTCTCTAC

81 V S H Q F A M G E R L N P Y A F A A L Y

+ 301 GGAAACCAACGATCTCTGCTCAGGTAAGCTGGACAAAGAGGGGCTCTCTGACAGA

101 G T N Q I F A Q G N L D N E G A L S T R

+ 361 TTCAACTACAGATGGGCTGACAGGACCATCAACAGACGAGTCTCTGATTTGGTGGGGC

121 F N Y R W G D R T I T K T Q F S I G G G

+ 421 CAGGATATGGCCGCTTTGACGATGAACACCTTTGGGACGACTTCAGTCTCCTCCTC

141 Q D M A Q F E H E H L G D D F S A S L K

+ 481 GCCATCAACCCCTCTTCTTGAAGGGCTCTCAAGGTATCTTTGTGCGGACTACCTC

161 A I N P S F L D G G L T G I F V G D Y L

+ 541 CAGGCGCTCATCCAGACTCGGCTCGGCTCTCAGGCGCTCTGCAACGTCAGGGTCTC

181 Q A V T P R L G L G L Q A V W Q R Q G L

+ 601 ACTAGGGCCCGGACACCGCTATCTCTACTTTGCGCGCTACAAAGCGGTGACTGGGT

201 T Q G P D T A I S Y F A R Y K A G D W V

+ 661 GCTAGCGCTCAGCTCCAGGCTCAGGCTGCTCTCAACACTTCTCTGGAAGAAGCTGAGC

221 A S A Q L Q A Q G A L N T S F W K K L T

+ 721 GATAGGGTCAAGCTGGTGTGATATGACGCTATCTGTCTGCTCCTCAGACATGATG

241 D R V Q A G V D M T L S V A P S C S M M

+ 781 GGTGGCTTACCAAGGAAGGCATCAACCTTTGGTCCAAAGTACGACTTCAGATGTC

261 G G L T K E G I T T F G A K Y D F R M S

+ 841 ACCTTCAGGCTCAGATCGACTCCAAAGGCAAGCTCAGCTGCTTGTCTGAGAAGCGTCTT

281 T F R A Q I D S K G K L S C L L E K R L

+ 901 GGTGCGCCCGCTCAGCTTCACTTCACTGCTGATGTTGACACGCTCACTCAACAGCC

301 G A A P V T L T F A A D V D H V T Q A

+ 961 AAGCTCGGATGTCGCTTCACTTGAAGGCTGATGTGATCTCCAGGAGCAGCAAGAG

321 K L G M S V S I E A S D V D L Q E Q Q E

+1021 GGTGCCAGTCCCTCAACATCCCTTTTAACTCGAATATGTATTTGGGATGAAGAC

341 G A Q S L N I P F *

+1081 ACTGGTCAATGGGCGACATTTGGTTTCGCTCCACCTTGCACCTCTACTCATTGTGTT

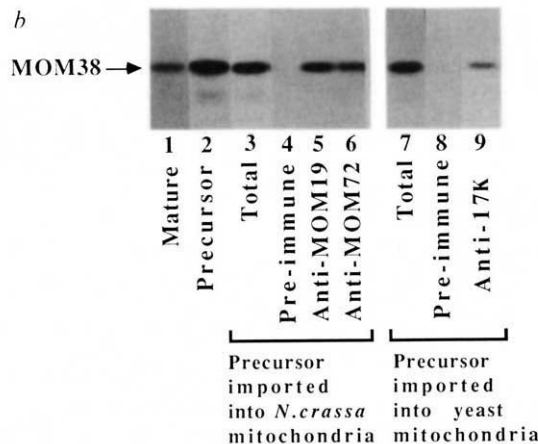
+1141 GACAAAGTAACTCAATCACACAGATGCCACCTTTGTAACCAAGGAACAGAAAGGGTGGG

+1201 GTCATACCTTTTCACGCTCCCTGCTACACGCGCAATTTCTGTTTGAACCATCTTCAT

+1261 CACATCATCTAGTACAGCTGGTCAAGCGAGTGTGCTGCGGAAATCGAAAGTGGAC

+1321 GTTGGAGACCTCCCATCGAATGGTTCGCGATGGCTTGGGTCTTTGCATACAGTTTCA

+1381 CGGACGATAGACATCTCTACACATTTTCCACCTTAAAAAAG



antibody screening and subcloned into pGEM4 (Promega Biotec)⁴⁴. The cDNA insert was sequenced using internal restriction sites and MOM38-specific oligonucleotide primers. Both strands were sequenced overlapping at least three times. A possible membrane-spanning sequence is underlined. Additional uncharged segments are found at amino-acid positions 72–88, 219–236 and 249–264. Boxes indicate the A and B regions of a putative ATP-binding domain³⁰. b, The precursor of *N. crassa* MOM38 is imported and assembled in isolated *N. crassa* and yeast mitochondria. Lane 1 shows MOM38 immunoprecipitated from ³⁵S-labelled *N. crassa* mitochondria. The MOM38 precursor was synthesized in reticulocyte lysate in the presence of [³⁵S]methionine by coupled transcription/translation¹⁸ (lane 2) (the precursor was efficiently recognized by anti-MOM38 antibodies). The reticulocyte lysate was then incubated with isolated mitochondria from *N. crassa* (lanes 3–6) or *Saccharomyces cerevisiae*⁴⁵ (lanes 7–9) in BSA buffer (see legend to Fig. 1) for 20 min at 25 °C. Samples 3 and 7 were treated with proteinase K (20 μ g ml⁻¹) for 20 min at 0 °C and the mitochondria reisolated. The mitochondria of samples 4–6, 8 and 9 were reisolated, lysed in digitonin buffer (see legend to Fig. 1) and immunoprecipitated with the indicated antibodies. Samples were analysed by SDS-PAGE and fluorography. The 17K protein is an outer membrane protein of yeast mitochondria⁴⁰ (the putative homologue to *N. crassa* MOM19). The precursor of MOM38 in reticulocyte lysate was completely digested by low concentrations of trypsin (20 μ g ml⁻¹) and proteinase K (10 μ g ml⁻¹), in contrast to the protein imported into mitochondria.

FIG. 5 Primary sequence of MOM38 and assembly of the precursor *in vitro*. a, Nucleotide sequence of the cDNA coding for *N. crassa* MOM38 and derived amino-acid sequence (single letter code). A full-length cDNA clone coding for MOM38 was isolated from a λ gt11 library of *N. crassa* cDNA²⁹ by

(residues 146–218) that exhibits 68% similarity (including 25% identity) to a sequence (residues 66–136) of the bovine mitochondrial phosphate carrier protein, an inner membrane protein³³. Homology is also found to the similar region of the yeast mitochondrial phosphate carrier protein³⁴. Furthermore, a sequence of about 60 amino-acid residues (residues 288–346 of MOM38) shows 78% similarity (including 35% identity) to a sequence (residues 522–584) of the *czcA* gene product of the divalent cation efflux system from the bacterium *Alcaligenes eutrophus*³⁵. As these sequences are present in membrane proteins with transport function, a role for structure and/or function of membrane carrier proteins might be possible.

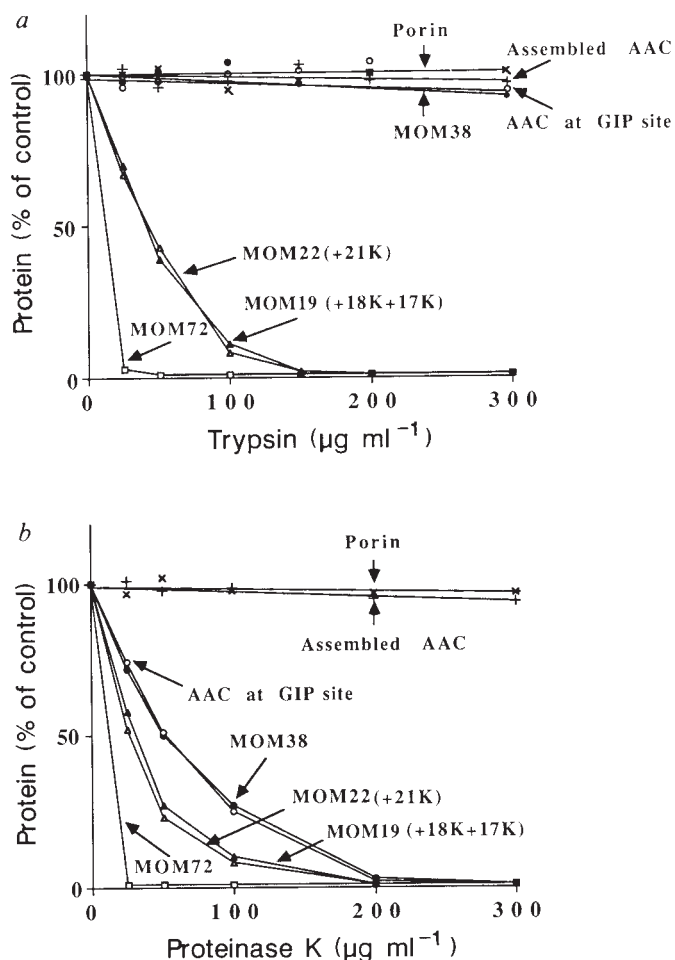
The precursor of MOM38 was synthesized in rabbit reticulocyte lysate by coupled transcription/translation. As expected for an outer membrane protein, the precursor exhibited the same apparent molecular size as the mature mitochondrial protein (Fig. 5b, lanes 1 and 2), indicating the absence of a cleavable targeting sequence. The precursor was efficiently imported into isolated *N. crassa* mitochondria (Fig. 5b, lane 3) and correctly assembled into the receptor complex as shown by the specific coprecipitation with anti-MOM19 and anti-MOM72 antibodies (Fig. 5b, lanes 5 and 6). Moreover, the precursor of *N. crassa* MOM38 was imported into isolated yeast mitochondria (Fig. 5b, lane 7) and could be coprecipitated with antibodies directed against a yeast mitochondrial outer membrane protein of 17K¹⁰ (Fig. 5b, lane 9), the putative homologue of MOM19 (H. F. Steger, T. S., N. P. and W. N., manuscript in preparation). This result suggests that a structurally and functionally equivalent receptor complex may exist in yeast mitochondria.

What is the function of MOM38 in protein import? We previously reported a characteristic feature of the GIP site,

namely its resistance to very high concentrations of trypsin whereas it is degraded at intermediate concentrations of proteinase K^{3,18,23,24,36}. In the following experiment (Fig. 6) we determined whether a component of the receptor complex showed this property. MOM19, MOM22 and MOM72 were digested by treatment of isolated mitochondria with trypsin. MOM38 was completely resistant to treatment with trypsin as was the precursor of ADP/ATP carrier accumulated at the GIP site (Fig. 6a). The precursor of ADP/ATP carrier itself was very sensitive to digestion by trypsin, both in reticulocyte lysate and on lysis of mitochondria with detergent²³, whereas MOM38 was also resistant to trypsin after lysis of mitochondria. Moreover, MOM38 in the outer membrane was not accessible to antibodies prepared against denatured MOM38 (data not shown). As the precursor of MOM38 in reticulocyte lysate is digested by trypsin and recognized by antibodies (see legend to Fig. 5), it is concluded that the imported MOM38 acquires a conformation in the outer membrane that does not allow access for trypsin or antibodies.

In summary, the GIP site copurifies with the receptor complex (Fig. 2a). Three of the complex components are digested at protease concentrations that do not degrade the GIP site, excluding them as possible candidates for GIP (under these conditions, the precursor of ADP/ATP carrier at the GIP-site remains protected and is fully competent for completion of import^{18,23,24}). With all likelihood, MOM38 is responsible for the protection of precursors accumulated at the GIP site and thus forms an essential part of GIP^{18,19}. Furthermore, when mitochondria were treated with proteinase K the sensitivity of MOM38 again correlated well with that of the GIP site (Fig. 6b). The function of MOM22 is unknown; its presence in the receptor complex and its exposure on the mitochondrial surface

FIG. 6 The protease sensitivities of GIP site and MOM38, but of no other component of the complex, show close correlation. Isolated *N. crassa* mitochondria (0.25 mg protein ml⁻¹) carrying the GIP intermediate of the ADP/ATP carrier (AAC) were treated with trypsin (a) or proteinase K (b) in BSA buffer (see legend to Fig. 1) as described²³. The mitochondria were reisolated and analysed for GIP-associated ADP/ATP carrier by SDS-PAGE and fluorography and for MOM38, MOM19 (and 18K and 17K fragments derived from MOM19 (ref. 9)), MOM22 (and a 21K fragment derived from MOM22), MOM72, assembled AAC, and porin by immunodecoration.



like the receptors MOM19 and MOM72 (Fig. 6) may suggest an involvement in initial steps of protein import.

Discussion

We report here that both mitochondrial protein import receptors, the master receptor MOM19 and the receptor for the ADP/ATP carrier (MOM72)⁹⁻¹¹, are present in a protein complex in the outer membrane of *N. crassa* mitochondria. Analysis of the constituents of the complex led to the identification of two new components, the outer membrane proteins MOM38 and MOM22. MOM38, an integral membrane protein, apparently represents the general insertion site GIP (or a part of it) which is responsible for the accumulation of precursor proteins in the outer membrane before their transfer into the inner membrane^{18,19}. This implies that the transfer of precursor proteins from the bound state at the receptors to the membrane-inserted GIP state occurs by way of direct interaction of the receptors with MOM38. MOM22, the fourth component of this receptor complex, is exposed on the outer membrane surface, yet its function is currently unknown.

Our results suggest the following working hypothesis. The association between MOM19 and MOM38 forms the core of the receptor complex. Precursor proteins that are recognized by MOM19 can thus be immediately donated to the GIP site, that is MOM38, and thereby be inserted into the outer membrane. MOM72 may assemble with and disassemble from this complex. The precursor of ADP/ATP carrier first binds to those MOM72 molecules that are not in the complex and thereby remains on the mitochondrial surface¹¹. Upon assembly of MOM72 into the complex, the precursor is donated to MOM38 and inserted into the outer membrane. The further transport of precursors occurs through contact sites between both mitochondrial membranes^{2,3,20,21,25-27}. As the constituents of the complex are not exclusively located in contact sites^{9,11}, lateral mobility of these constituents and/or contact sites in the outer membrane is implied. The association of the receptor complex with a fraction of precursors in transit through contact sites also suggests a role in membrane

translocation.

This model agrees well with results obtained with yeast mitochondria. The import receptors MOM72 (ref. 10) and MOM19 (H. F. Steger, T. S., N. P. and W. N., manuscript in preparation) have been identified in yeast. These components seem to be present in a complex similar to that of *N. crassa* as indicated by the observed ability of *N. crassa* MOM38 to assemble with the yeast components after import into isolated mitochondria. Vestweber *et al.*¹² have identified a protein of 42K in the yeast mitochondrial outer membrane by cross-linking to a precursor arrested in contact sites. The properties of this 42K protein, that is apparent size, association with a fraction of precursors trapped in contact sites¹², high resistance to trypsin³⁷, and distribution over the entire outer membrane¹², correlate with the expected properties of the yeast equivalent to MOM38. In fact, this 42K protein seems to be present in a receptor complex of yeast mitochondria (N. P., T. S., K. Dietmeier, H. F. Steger and W. N., unpublished results). We propose that the yeast 42K protein is the equivalent of MOM38 and thus represents the GIP of yeast mitochondria. The hypothesis of cycles of assembly and disassembly of components of the protein transport apparatus and of lateral mobility of the components in the membrane is thus supported by findings both with *N. crassa* and yeast. We speculate that similar dynamic behaviour may also be important for other membrane systems where recognition, insertion and translocation of precursor proteins takes place.

Note added in proof: *N. crassa* MOM38 shows 40% amino-acid sequence identity (plus 37% isofunctional amino-acid exchanges) with the 42K protein identified in yeast mitochondria (see accompanying article⁴⁶). The similarity extends over the entire lengths of the proteins (with the exception of 21 additional amino-acid residues at the N-terminus of the yeast protein). An identity of 40% is in the usual range observed with equivalent proteins of *N. crassa* and *S. cerevisiae* (for example, refs 10 and 29). This supports our proposal that the 42K protein represents the yeast mitochondrial general insertion site GIP or part of it. □

Received 8 October; accepted 26 October 1990.

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ACKNOWLEDGEMENTS. We thank C. Burkl, U. Hanemann, B. Steizle and A. Weinzierl for expert technical assistance, G. Arnold and I. Leitner for the synthesis of oligonucleotides, H. Feldmann and J. Thierack for help with computer analysis, H. Wohlrab for communicating results before publication, and C. Hergersberg, K. Trütsch and M. Tropschug for experimental advice. This study was supported by the Sonderforschungsbereich 184 (project B1), the Genzentrum München, the Fonds der Chemischen Industrie, and a fellowship to M.K. from the Boehringer Ingelheim Fonds.

Consistent neutrino masses from cosmology and solar physics

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THE solar neutrino problem and cosmological dark matter can both be accounted for by non-zero neutrino masses¹⁻⁵ that are broadly compatible with the so-called 'see-saw' relation, which comes from a simple theoretical model⁶ for the neutrino masses and according to which the mass of each neutrino type is proportional to the square of the mass of the associated quark. Recently both the solar and the cosmological estimates of neutrino masses have been considerably improved. New solar neutrino data⁷ lead to a more precise constraint^{8,9} on the masses of the electron and either the muon or tau neutrinos if the dearth of detected solar neutrinos is ascribed to resonantly enhanced mixing of neutrino types within the Sun (the MSW effect^{10,11}). In addition, if galactic H I ionization is a consequence of decaying tau neutrinos that constitute dark matter, a more precise estimate of the tau neutrino mass follows¹². Here I point out that these more accurate estimates still conform to the see-saw relation, a result which, unless it is a chance agreement, ties together three unproven hypotheses: the see-saw mechanism, the MSW effect and the decaying neutrino hypothesis.

It has recently been found¹³ that there are only three types of neutrino—the electron neutrino ν_e , the muon neutrino ν_μ and the tau neutrino ν_τ . The following discussion requires that these particles have non-zero rest masses, which has not yet been established. Present laboratory limits¹⁴ on these masses are $m_{\nu_e} < 8$ eV, $m_{\nu_\mu} < 250$ keV and $m_{\nu_\tau} < 35$ MeV.

The solar-neutrino problem¹⁵ is that fewer electron neutrinos from the Sun are detected than would be expected from the predictions of the standard solar model. The most attractive explanation of this anomaly is the so-called MSW effect^{10,11}, which involves ν_e oscillating into ν_μ and/or ν_τ at a rate resonantly enhanced by their interactions with the solar material. These oscillations require that the neutrinos have non-zero rest masses, and one of the important parameters governing the MSW effect is $\Delta m^2 = m_{\nu_\mu}^2 - m_{\nu_e}^2$ (or $m_{\nu_\tau}^2 - m_{\nu_e}^2$). The earlier analysis¹⁵ of the MSW effect indicated that the solar neutrino problem could be solved if

$$\Delta m^2 \approx 10^{-4} - 10^{-7} \text{ eV}^2 \quad (1)$$

The cosmological problem arises if one demands that the Universe possess the critical density¹⁶. This leads to a problem because, according to the standard analysis¹⁷ of the primordial synthesis of the light isotopes ^2H , ^3He , ^4He and ^7Li , baryons can contribute at most a few per cent of the critical density. Thus one would have to introduce dark matter to provide most of the critical density. If neutrinos have non-zero rest mass they are an attractive candidate for this dark matter because they would have been created by pair production in the early stages of the hot Big Bang, and would have survived until today. It is, however, controversial¹⁸ whether N -body calculations of galaxy formation in the presence of massive neutrinos can lead to the observed small-scale distribution of galaxies. More work is needed to resolve this question, but for the time being we may continue to consider the possibility that the dark matter consists of massive neutrinos.

The present cosmological concentration of each neutrino type must in any case be 3/11 of the photon concentration in the cosmic microwave background ($\sim 110 \text{ cm}^{-3}$). These neutrinos would possess the critical density if their masses were such that $\sum_i m_{\nu_i} \approx 100 h^2 \text{ eV}$ (ref. 15), where the sum is over the three neutrino types and h is related to the Hubble constant H by

$H = 100 h \text{ km s}^{-1} \text{ Mpc}^{-1}$. The observed value of h is uncertain to a factor of about two, $\frac{1}{2} \leq h \leq 1$.

I now consider a possible theoretical relation between m_{ν_e} , m_{ν_μ} and m_{ν_τ} . There is no generally accepted theory for the origin of neutrino rest masses (indeed, as mentioned above, it is not yet established whether any of the rest masses are in fact non-zero). An attractive possibility is the see-saw model⁶. In this model a matrix governs both the masses of the neutrinos and a large cut-off mass M , such as the grand unification mass at which the weak, electromagnetic and strong interactions become equal. According to one version of this model, when the mass matrix is diagonalized $m_{\nu_e} : m_{\nu_\mu} : m_{\nu_\tau} = m_u^2 / M : m_c^2 / M : m_t^2 / M$, where u , c and t refer to the up, charm and top quarks. The values of the quark masses are somewhat model-dependent, but the standard values¹⁹ are $m_u \approx 4$ MeV and $m_c \approx 1.1$ GeV. The mass of the top quark is more uncertain, but a current estimate¹⁴ is $m_t \approx 150$ GeV. In this case, $m_{\nu_e} : m_{\nu_\mu} : m_{\nu_\tau} = 1 : 7.5 \times 10^4 : 1.5 \times 10^9$. Likely values of M fall into three possible categories¹⁻⁵: (1) A large value of the order of 10^{15} GeV. (2) An intermediate value of the order of $10^{11} - 10^{12}$ GeV. (3) A low value ($\ll 1$ GeV).

Clearly, if this version of the see-saw mechanism is correct, and if neutrinos provide the critical density, then ν_τ is by far the most massive neutrino type and

$$m_{\nu_\tau} \approx 100 h^2 \text{ eV} \quad (2)$$

It would then follow that $M \approx 2 \times 10^{11} h^{-2} \text{ GeV}$, corresponding to the intermediate case. Further, $m_{\nu_\mu} \approx 5 \times 10^{-3} h^2 \text{ eV}$, $m_{\nu_e} \approx 7 \times 10^{-8} h^2 \text{ eV}$, and $m_{\nu_\mu}^2 - m_{\nu_e}^2 \approx 25 \times 10^{-26} h^4 \text{ eV}^2$, which falls in the range (1). This broad consistency of the various assumptions involved has been widely recognized¹⁻⁵.

Bahcall and Bethe⁸ have recently reduced the permitted range of Δm^2 by comparing the results of the Kamiokande solar neutrino/electron scattering experiment⁷ with those from the chlorine experiment²⁰. They find

$$\Delta m^2 \approx 10^{-6} \text{ eV}^2 \quad (3)$$

for which they claim an uncertainty of less than an order of magnitude.

The estimate of m_{ν_τ} has also recently been sharpened if one accepts a speculative theory^{12,21,22} according to which tau neutrinos decay into hydrogen-ionizing photons at a rate $\approx 10^{-23} \text{ s}^{-1}$. The energy E_γ of the decay photon in the rest frame of the parent neutrino is

$$E_\gamma = \frac{m_{\nu_\tau}^2 - m_{\nu_\mu}^2}{2m_{\nu_\tau}} \quad (4)$$

If the see-saw mechanism is correct $m_{\nu_\mu} \ll m_{\nu_\tau}$, and then $E_\gamma \approx \frac{1}{2} m_{\nu_\tau}$. As I discuss elsewhere^{12,21-23} there are astronomical observations to constrain both E_γ and m_{ν_τ} . These constraints arise from ultraviolet observations of the Coma cluster of galaxies, from H α observations of an intergalactic H I cloud in Leo, and from an application of the Liouville theorem to the tau neutrinos assumed to dominate our Galaxy. I obtain

$$m_{\nu_\tau} = 29 \pm 1 \text{ eV} \quad (5)$$

which is compatible with result (2) if $h \approx 0.55$. I also find that $E_\gamma \approx \frac{1}{2} m_{\nu_\tau}$ with a precision implying, from result (4), that $m_{\nu_\mu} \leq 5 \text{ eV}$.

Combining result (5) with the version of the see-saw mechanism used above implies $m_{\nu_\mu} \approx 1.5 \times 10^{-3} \text{ eV}$ and $m_{\nu_e} \approx 2 \times 10^{-8} \text{ eV}$, so that Δm^2 agrees with the Bahcall-Bethe result (3), within their claimed uncertainty.

I conclude that the see-saw mechanism, the MSW effect and the decaying neutrino hypothesis form, so far, a consistent pattern. A further test²⁴ of result (5) would be the experimental discovery of $\nu_\mu - \nu_\tau$ oscillations. There are proposals¹⁴ from both CERN and Fermilab to carry out such experiments. The value of m_{ν_τ} might also be determined by time-of-flight considerations

if neutrinos emitted by a nearby supernova were detected by the next generation of water Cerenkov detectors²⁵. Searches are also being made for the proposed photon line emitted by decaying tau neutrinos in various regions of the Universe. Because the line emitted by most sources would be expected to be rather narrow, the quantity E_γ could be determined with considerable precision if the line itself were observed. This would then lead to an accurate value of m_{ν_τ} , if the ideas described here are correct. $\square$

Received 3 September; accepted 9 November 1990.

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ACKNOWLEDGEMENTS. I thank the Ministero Italiano per la Ricerca Scientifica e Tecnologica for financial support.

Discovery of hotspots on Io using disk-resolved infrared imaging

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ACTIVE, tidally driven volcanism on Jupiter's satellite Io^{1,2} was discovered in Voyager images in 1979. Voyager also detected the thermal emission from the volcanoes^{3,4}, and this emission has since been monitored from Earth using a variety of techniques employing single-element infrared detectors^{5–10}. Because of Io's small angular size (no more than 1.2 arcsec), however, it has normally been possible to resolve and locate only the brightest individual hotspot, Loki, although additional hotspots can be located at five-year intervals during occultations of Io by the other galilean satellites^{11,12}. Here we present first results using two new techniques for ground-based observation of Io's hotspots. First, use of an infrared array camera allowed us to exploit the Mauna Kea Observatory's excellent atmospheric stability to obtain direct infrared images of Io with resolution better than 0.5 arcsec. This allowed us to see more than one hotspot on Io in Jupiter eclipse (in Jupiter's shadow). Second, we used the camera to make the first observations of the Jupiter occultation (disappearance behind Jupiter) of the hotspots. These new techniques have revealed and located at least three hotspots, and will now permit routine ground-based monitoring of the locations, temperatures and sizes of multiple hotspots on Io.

We obtained 1.6–4.8 μm wavelength images of Io in sunlight and in Jupiter eclipse using the 3.2-m NASA Infrared Telescope Facility (IRTF) and its 62×58 pixel InSb array camera, ProtoCAM¹³, on 22–24 December 1989 and 21 March 1990, with an image scale of 0.136 arcsec pixel⁻¹. The full width at half maximum (FWHM) of star images at wavelengths greater than 3 μm was 0.50–0.35 arcsec on both runs, approaching the diffraction limited FWHM of 0.28 arcsec at 3.8 μm , so that Io's disk (diameter 1.2 arcsec in December and 1.0 arcsec in March) was easily resolved (Fig. 1).

The 3.8- μm images taken in December 1989 (Fig. 1a) show thermal emission from a bright hotspot against the sunlit disk of Io. Location on the images and Jupiter occultation timings (see below) indicate that this hotspot is near the dominant volcano Loki discovered by Voyager³, and we refer to it as Loki-A. Loki-A was also conspicuous at 4.8 μm , where it was brighter with respect to the sunlit disk. In March 1990 this spot had faded dramatically and was no longer visible in sunlight (Fig. 1b).

We also imaged Io in eclipse by Jupiter. Loki-A dominated the December eclipse images (Fig. 1c), but in March it was replaced by a much fainter nearby source, which we call Loki-B (Fig. 1d). In both months, the high spatial resolution also allowed us to see another hotspot. The second spot was almost constant in flux (see below), and we gave it the informal name Kanehekili (a Hawaiian thunder god¹⁴).

We observed the Jupiter occultation ingress (in December) and egress (in March) of the hotspots in Jupiter eclipse, taking 1-s exposures every 2.1–3.5 s through a 3.8- μm broadband filter in which Jupiter is dark owing to strong atmospheric CH₄ absorption. After the image of Jupiter is digitally removed from each frame, the photometric quality is excellent, with a frame-to-frame signal-to-noise ratio of 150 in the December ingress light curves (Fig. 2). The absolute fluxes were derived using standard IRTF photometric reduction and absolute calibration techniques¹⁵.

The disappearance of Kanehekili behind Jupiter is clearly seen in the December ingress light curve (Fig. 2, top), followed by the much brighter Loki-A. In the March occultation egress (Fig. 2, bottom), at least three separate hotspots are seen emerging in succession. The first is the faintest Io hotspot yet seen from Earth. The second is apparently Kanehekili and the third is Loki-B.

For a source on Io occulted by Jupiter's atmosphere, 50% light loss due to differential refraction is expected at a pressure of 2.2 ± 0.5 mbar (ref. 16), assuming a temperature of 160 ± 10 K (ref. 17) and a He mass fraction of 0.20 ± 0.05 (ref. 18). Synthetic transmission spectra (G. Bjoraker, personal communication) indicate that a grazing incidence ray at this level should suffer 12% CH₄ absorption integrated over our filter bandpass. Observations of Ganymede eclipses by Jupiter¹⁹ show that haze absorption is negligible at this level. Refraction thus dominates the light loss, and Fig. 2 shows extinction curves expected from refraction of point sources. In the December 1989 curve, Loki-A fades as rapidly as a point source at our time resolution and is therefore not resolved. This places a preliminary upper limit of ~ 200 km on the extent of the 3.8- μm -emitting region perpendicular to Jupiter's limb, implying a source radius of ≤ 120 km

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TABLE 1 Hotspot data

Hotspot or Date	Loki-A 24 December 1989	Loki-B 21 March 1990	Kanehekili 24 December 1989	Kanehekili 21 March 1990	Faint spot 21 March 1990
Position					
Latitude	+35±4	+10±6	-13±10	-22±2	-15±30
Longitude (W)	309±2	310±6	38±3	37±2	78±12
Vertical flux (GW sr ⁻¹ μm ⁻¹)					
3.8-μm occultation*	260±26	22±4	18±3	18±2	≥7.5±1.3†
3.8-μm regional‡	—	31±3	—	26±3	—
4.8-μm regional‡	—	73±7	—	45±5	—
Temperature (K)	≥370§	395±35	—	460±35	—
Radius, if circular (km)	≤120§	31 ⁺¹⁶ ₋₉	—	14 ⁺⁵ ₋₃	—

The positions give the maximum extent of error boxes (see Fig. 3). The position of Loki-A is from Jupiter occultation ingress and egress, assuming timing of the latter is reliable. The position of Loki-B is from Jupiter occultation egress and offset from Kanehekili. The position of Kanehekili in December 1989 is from Jupiter occultation ingress and offset from Loki-A, and in March 1990 is from the December 1989 position and the March 1990 occultation egress, assuming its location is fixed. The position of the faint spot is a one-dimensional constraint (see Fig. 3). The vertical flux has been divided by the cosine of emission angle to correct for viewing geometry. The radius and temperature of Loki-A are from duration of occultation ingress, and from the duration of ingress and 3.8-μm flux, respectively. The temperature of Loki-B and Kanehekili are from the ratio of the regional 3.8-μm and 4.8-μm fluxes. The radii of Loki-B and Kanehekili were deduced from the temperature and the regional 3.8-μm flux.

* Sudden flux change when source is occulted; excludes contribution of smaller sources in same region.

† Lower limit to vertical flux, as only lower limit available for emission angle.

‡ Total flux in eclipse images, divided between Loki-B and Kanehekili in ratio of their peak intensities. Corrected to vertical flux assuming all flux is at the two major sources.

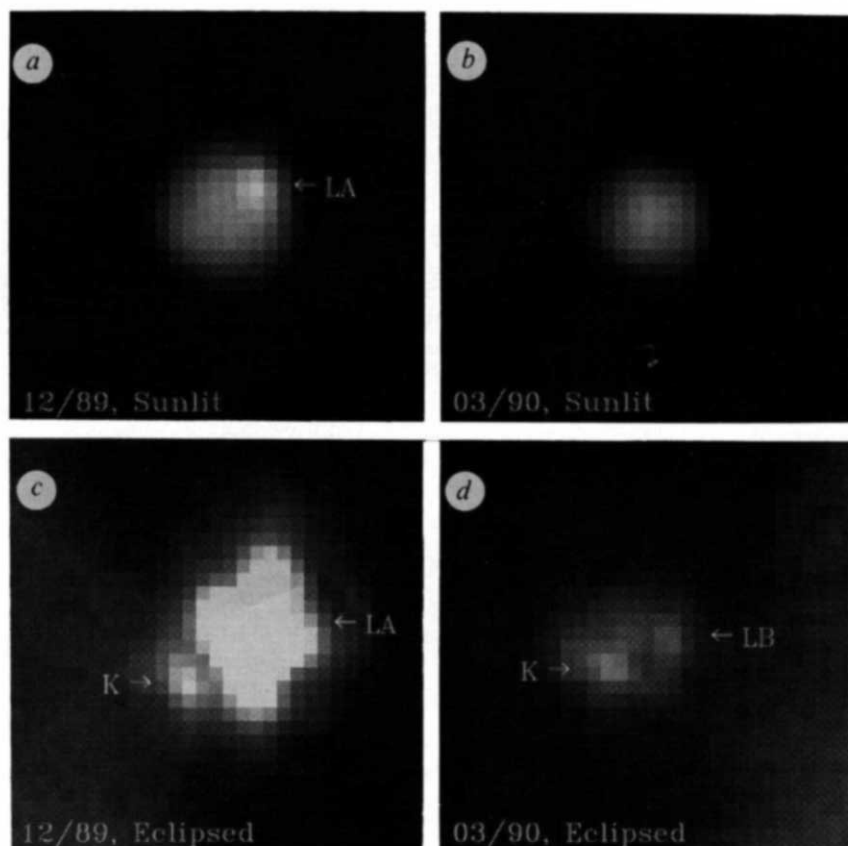
if it is circular. The lack of an extended refractive tail may be due to CH₄ absorption deeper in Jupiter's atmosphere.

A single occultation timing locates a hotspot in one dimension with a maximum precision of about 50 km. Hotspot data are summarized in Table 1 and positions are shown in Fig. 3. Positions are derived from occultation timings using the Jet Propulsion Laboratory galilean satellite ephemeris E3gl (ref. 11), Io rotational parameters²⁰ and the shape and size of Jupiter at the 2.2-mbar level¹⁷. A match, to better than ±2 s, between the preceding eclipse ingress light curve and ephemeris predictions confirms the accuracy of both the ephemeris and the timing

of the December occultation ingress. Further positional constraints come from the relative positions of the hotspots in the images, which have estimated errors, based on repeatability between images, of ±0.3 pixels (140 km on Io). The measured relative positions are consistent with the occultation timings to this precision.

In December 1989 Loki-A was bright enough that its Jupiter occultation egress (in sunlight) could also be timed, giving a two-dimensional position. Because this was an experimental run and we have no independent check on the egress timing, and because the inferred position is 16° N of Loki's 1985 location^{11,12}

FIG. 1 Images of Io at 3.8 μm, pixel size 0.136 arcsec. *a*, 24 December 1989, diameter=1.2 arcsec, central Io longitude $\theta=349^\circ$. Resolved sunlit disk of Io plus volcanic thermal emission from Loki-A (LA, upper right). *b*, 21 March 1990, diameter=1.0 arcsec, $\theta=349^\circ$. Loki-A has faded and is invisible against the sunlit disk. *c*, 24 December 1989, $\theta=350^\circ$, in eclipse, Jupiter on left. Processing has saturated the Loki-A image to show a second hotspot, Kanehekili (K), 0.9 arcsec away. Cross shape of outer part of Loki-A image is due to imperfections in the IRTF optics. *d*, 21 March 1990, $\theta=10^\circ$, in eclipse, Jupiter on right. Processing comparable with *c*, showing the dramatic fading of Loki-A. The much fainter nearby source Loki-B (LB) is visible instead. Fading is due to a combination of an intrinsic drop in flux and the different orientation of Io. Kanehekili (K), 0.65 arcsec away, is now the brightest source.



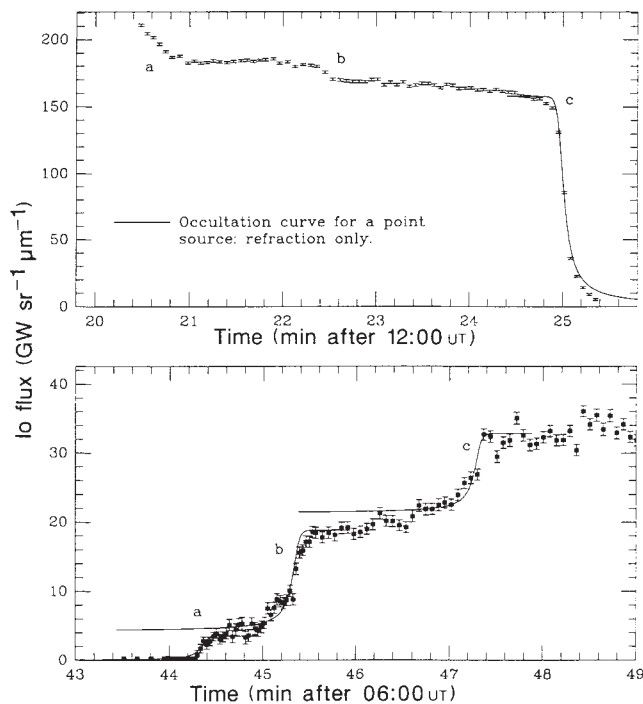


FIG. 2 3.8- μm Jupiter occultation light curves of Io in eclipse. Top, occultation ingress, 24 December 1989. Notable events are (a) completion of eclipse ingress, (b) occultation of Kanehekili, (c) occultation of Loki-A. Bottom, occultation egress, 21 March 1990. Volcanic emission is much fainter than in December 1989, accounting for poorer signal to noise. Notable events are: (a) appearance of a very faint hotspot, (b) appearance of Kanehekili, (c) appearance of Loki-B. The poor fit to the expected point-source refractive extinction curves suggests that several other sources are also present.

and even farther north of the March 1990 Loki-B position (Fig. 3), we regard the Loki-A position as tentative. In future occultation observations with reliable timing, however, high-precision two-dimensional positions of bright hotspots such as Loki-A should be routinely available.

Kanehekili's location is constrained in a N-S direction by its position relative to Loki-A in the December images. Assuming the Loki-A position in Table 1 is reliable, the uncertainties in the Kanehekili December position just overlap with the March occultation egress positional constraint (Fig. 3). Therefore if Kanehekili was stationary between December and March (as suggested by its constant flux) its location is very tightly constrained to be within the overlap area. If it moved, its position at each time is much less certain.

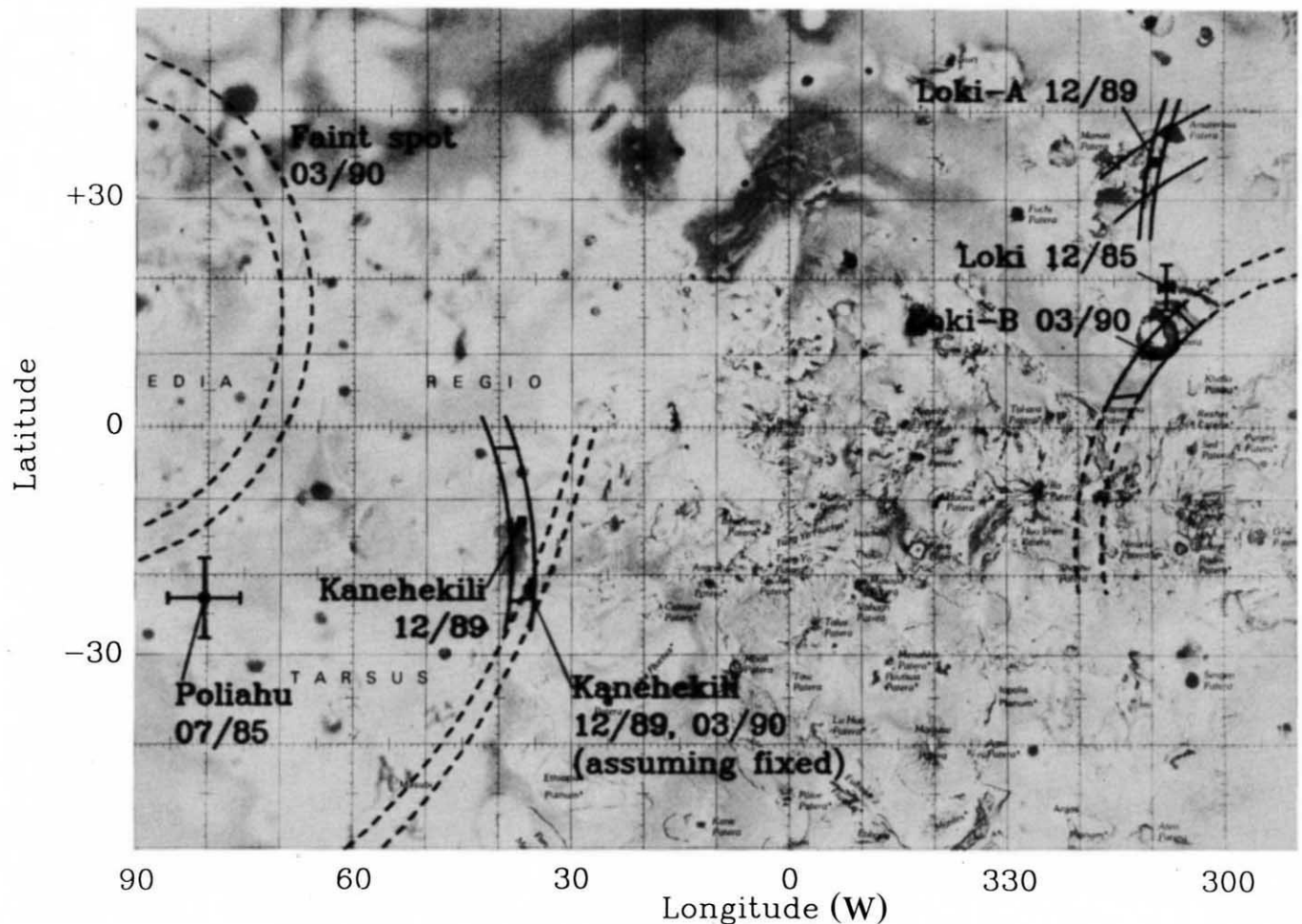


FIG. 3 Map of Io showing hotspot location constraints. Curved lines (solid, 24 December 1989; dashed, 21 March 1990) show uncertainty limits of projection of Jupiter's limb on Io at hotspot occultation ingress or egress times; hotspots must lie within these bands. Only the relevant parts of the bands are shown. The relative positions of Loki-B and Kanehekili in the 21

March 1990 images, and the requirement that both hotspots must lie on Io's disk, limits the extent of their respective bands. Boxes within two of the bands show additional constraints based on relative hotspot positions in the images (see text). Positions of Loki on 25 December 1985 and Poliahu on 10 July 1985 from Goguen *et al.*^{11,12}

The position of Loki-B in March is constrained in one dimension by its occultation egress time: the constraint in the other dimension is based on its position in the images relative to Kanehekili and the assumption that Kanehekili was fixed. The resulting error box includes Loki Patera, a prominent dark lake-like feature seen by Voyager, lending some support to this reasoning.

A bright hotspot, presumably Loki-A, was also seen in speckle observations by Howell (personal communication), polarization measurements made by some of us in November and December (Goguen and Sinton), and in eclipse observations on 8 November 1989 (Spencer and Sinton). If Loki-A was actually in the same location as Loki-B, at or near Loki Patera, its 3.8- μm flux dropped by a factor of 10 between December 1989 and March 1990. But if our tentative position for Loki-A is correct, it faded below detectability by March 1990. Our tentative location places it close to Amaterasu Patera, identified as a hotspot by Voyager in 1979⁴. In December it may have masked the much fainter lower-latitude emission from Loki-B. Loki-A's 3.8- μm December flux is comparable with previous observations of Loki at its brightest⁵. A similar dramatic fading of a hotspot in the Loki region occurred between December 1985 and September 1986¹⁰.

In contrast, Kanehekili's 3.8- μm flux was virtually the same in December and March, indicating a different type of volcanic activity. The December error box, but not the position assuming no motion between December and March, includes a conspicuous unnamed dark rectangular area of flows imaged (but not scanned in the infrared) by Voyager.

The third, very faint, hotspot seen in the March occultation egress is farther west, in a region seen only at low resolution by Voyager (Fig. 3). Because its position is only constrained in one dimension it is not yet possible to correlate it with any specific feature seen by Voyager. Its error 'box' just excludes the location of the short-lived source Poliahu seen on 10 July 1985^{11,12}.

The 24 December 1989 temperature of Loki-A is constrained to be ≥ 370 K by its 3.8- μm flux and the fact that its radius is ≤ 120 km, if circular. Because all occultation observations are at 3.8 μm , the only direct temperature measurements come from post-occultation images taken in March in which Loki and Kanehekili are resolved at both 3.8 μm (Fig. 1d) and 4.8 μm . Colour temperatures (Table 1) are within the normal range for Io hotspots⁵, and Kanehekili is probably somewhat hotter than Loki-B. The 2.2- μm eclipse images taken in March do not resolve individual hotspots but indicate small areas with temperatures over 600 K somewhere on the disk, as did disk-integrated eclipse measurements at this wavelength in 1979²¹. □

Imaging C₆₀ clusters on a surface using a scanning tunnelling microscope

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RECENT developments in the synthesis of macroscopic amounts of the C₆₀ and C₇₀ carbon clusters¹⁻³ have made possible spectroscopic^{1,2,4-7} studies of these species. Infrared^{1,2} and vibrational Raman⁴ spectra strongly support the proposed 'soccer ball' structure for the C₆₀ cluster⁸, and NMR spectroscopy directly confirms its icosahedral symmetry^{5,6}. Here we present images of C₆₀ clusters obtained with the scanning tunnelling microscope. These species are roughly spherical and form mobile hexagonal arrays on a Au(111) surface with an intercluster spacing of 11.0 Å. Brighter features evident in the arrays are tentatively identified as C₇₀ clusters.

We prepared samples of C₆₀ with some C₇₀ by resistively heating graphite in 100 torr of helium and subliming these clusters out of the resulting soot onto a gold foil. Mass spectrometry, vibrational Raman and NMR spectroscopy indicate that this method produces clean mixtures of C₆₀ and C₇₀ clusters^{4,5}. The C₇₀ NMR spectrum had five lines with intensity ratios 1:2:1:2:1 and chemical shifts 130.8, 145.3, 147.3, 148.1 and 150.6 p.p.m. (internally referenced to the C1 resonance of fully deuterated toluene at 137.5 p.p.m.), in agreement with measurements of Taylor *et al.*⁶. This five-line spectrum supports a proposed elongated 'rugby ball' shape for C₇₀ (ref. 9). The scanning tunnelling microscopy (STM) measurements were made in a ultra-high-vacuum system which has been described previously¹⁰. The gold foil with the fullerene deposit was transferred into this system through an airlock and left for several hours. A gold(111) single-crystal face was prepared by cycles of sputtering and annealing at 600 °C, as in previous studies¹¹⁻¹³. The clusters were transferred onto the crystal by placing the fullerene-coated foil 3 cm away and flashing it to 800 °C. The coated crystal had roughly equal Auger intensities for the 272-eV carbon peak and the 255-eV Au peak, indicating submonolayer fullerene coverage.

The crystal was then transferred (under ultra-high-vacuum conditions) to the STM chamber and slow-scan (1 line per second) images were recorded at a tunnel current of 0.3 nA. Figure 1 shows a series of images recorded a few minutes apart at a tip bias of -0.1 V. In Fig. 1a the slow upward scan sweeps the tip across an ordered array of numerous similar dots which we believe are C₆₀ ('fullerene') molecules. As the tip moves into the upper region of the frame, the surface steps down and the image becomes unstable. Some of the clusters appear distinctly brighter. In Fig. 1b, the initial tip position was translated away from this unstable region, but now instability and a large asperity appear in the right portion of the frame. A triad of brighter dots in the centre of this frame serves as a landmark. Figure 1c shows that the instabilities reappear if we translate back toward the top of the image. In Fig. 1d instrumental drift has subsided and the hexagonal character of the cluster arrays is apparent. In these arrays we find an intercluster spacing of 11.0 ± 0.5 Å (calibrated using the Au(111) atomic spacing¹¹), in reasonable agreement with an estimate obtained by adding the 7.1-Å diameter calculated for fullerene^{14,15} to the 3.35-Å layer separation of graphite. The straight rows of clusters are nearly parallel to the

Received 24 August; accepted 23 October 1990.

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ACKNOWLEDGEMENTS. The NASA Infrared Telescope Facility is operated by the University of Hawaii under contract with NASA.

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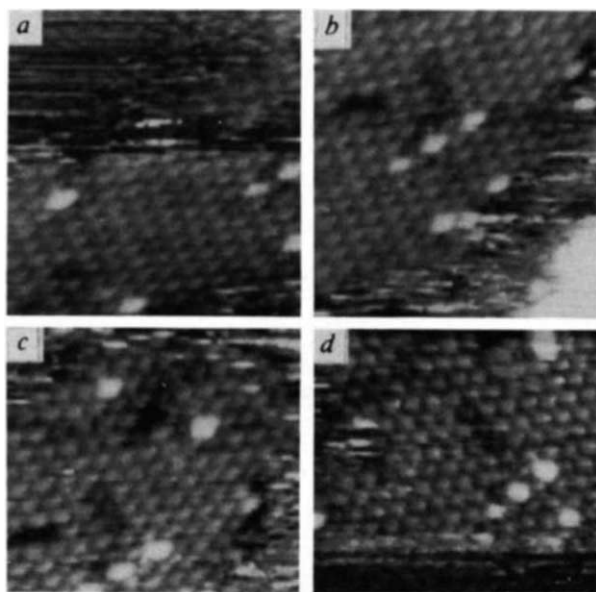


FIG. 1 Sequence of four STM images taken several minutes apart showing ordered arrangements of C_{60} clusters on a Au(111) surface. The brighter dots are believed to be due to C_{70} clusters. Bilinear interpolation has been used in rendering the raw data. The fast scan width (horizontal axis) is ~ 150 Å and the slow scan width varies owing to piezoelectric creep following translation of the tip to a new area.

Au atomic rows, so the array may be commensurate. In Fig. 1d, the vacancy above and to the left of the three bright dots has been filled in, and the bright dot at the lower right has moved.

Our data show ordered arrays of equivalent mobile species on the Au(111) surface with a spacing consistent with fullerene diameter. It is highly unlikely that the chemically unreactive gold surface can dissociate fullerene, especially if the fragments must be equivalent subunits that form an array with the proper spacing. We therefore conclude that these images represent intact fullerene clusters on the gold surface.

The observation of distinctly brighter dots in the ordered arrays (with an apparent height 2 Å greater than for the C_{60} clusters) demands an explanation. Conceivably the taller features are second-layer fullerene clusters. We would not, however, expect on-top registration for van der Waals' spheres, particularly as the STM tip would probably dislodge molecules in thicker layers^{16,17}. Here the taller features seem to be quite stable, even at the island edges. The most likely explanation is that the brighter dots are C_{70} clusters. The elongation of the C_{70} molecule, together with differences in its electronic structure relative to C_{60} (ref. 18), could easily account for the brighter clusters. Systematic work on samples with varying proportions of C_{60} and C_{70} is required to substantiate this assignment.

It may seem surprising that STM can image such a thick molecule which, by virtue of the 1.5 – 2.0 eV gap between its highest occupied molecular orbital (HOMO) and lowest unfilled molecular orbital (LUMO)¹⁸, might be thought of as insulating. But the C_{60} ionization potential is in the range 7.5 – 7.72 eV (ref. 19), so the LUMO is not far from the metal Fermi level (E_F). Furthermore, interactions with the Au surface and neighbouring clusters will shift, split and broaden the cluster orbitals thus giving some density of states at E_F , $\rho(E_F)$ (ref. 16). STM is quite sensitive to $\rho(E_F)$ on the topmost atoms in the cluster, because the tunnel current varies roughly exponentially as $\rho(E_F) 10^{\sqrt{\phi z}/2}$, where ϕ is the tunnel barrier in electron volts and z is the elevation of the top of the cluster in angstroms²⁰. For STM on metal surfaces in vacuum, ϕ is typically 1 eV, so a reduction of $\rho(E_F)$ by a factor of 10 relative to the metal only reduces the apparent height of the molecule by 2 Å. Experimentally the molecules are easily detected, as the corrugation in close-packed regions is ~ 1.5 Å and the peak-to-vacancy corrugation, or apparent height, is ~ 4 Å for fullerene and 6 Å for the taller clusters. The same ordered arrays and two heights of peaks are

observed at bias voltages between -1 and 1 V, so orbitals with sharply defined energies do not seem to be involved.

A potential difficulty for STM is the fact that the tip, which moves roughly 10 Å above the bare metal surface, withdraws only another 4 Å when traversing the molecules. Given the cluster diameter of ~ 10 Å, the tip approaches them very closely and may cause them to move. Indeed, in this system the observed image noise in areas bordering close-packed islands may indicate the presence of STM-induced²¹ or thermal motion of molecules not constrained by their neighbours. This behaviour may be relevant to earlier unsuccessful attempts to image these clusters using STM²².

We have not yet observed internal structure of individual fullerenes. These nearly spherical C_{60} clusters, which interact with the surface and each other only through van der Waals' interactions, may occupy rotational states which might smear out the atomic detail. Reducing the temperature or using a more reactive substrate could hinder such rotations¹⁷. The ease with which images of these carbon clusters can be made suggests that STM studies of these species and their derivatives²³ should be interesting and revealing. □

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ACKNOWLEDGEMENTS. We thank C. T. Rettner for helpful discussions.

Scanning tunnelling microscopy of solid C_{60}/C_{70}

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THE detection of a strong peak in mass spectra of carbon vapour corresponding to a cluster of 60 carbon atoms¹ led to the proposal that these clusters corresponded to highly symmetric C_{60} molecules in the shape of truncated icosahedra^{1,2}. Recently macroscopic quantities of C_{60} and the related species C_{70} (thought to have an elongated cage structure) have been prepared^{3,4} and purified in a solid form, called fullerite⁵. The availability of a solid form has made possible studies using infrared and ultraviolet-visible absorption spectroscopy, X-ray and electron diffraction⁵, NMR and Raman spectroscopy, all of which provide evidence that supports the proposed 'soccer ball' structure of C_{60} . Here we report the direct imaging of C_{60} molecules in sub-micrometre-sized particles of fullerite, using scanning tunnelling microscopy. The images reveal spherical molecules, spaced about 1 nm apart, stacked in close-packed arrays. Our results give a direct demonstration of the highly symmetric structure of these clusters.

We made fullerite using the two-step procedure described in ref. 5. The first step consists of vaporizing graphite rods in an atmosphere of ~100 torr of helium and harvesting the sooty carbon, which contains 5–10% C_{60} and C_{70} . The fullerite is then extracted from the soot using, for example, benzene, and a residue of polymer material is driven off by heating the product in an atmosphere of helium. The resulting powder was placed in an open-topped silica crucible and heated with an external tungsten heater to sublime the material onto gold-coated glass substrates positioned a few centimetres above the top of the crucible. We prepared samples at sublimation pressures of

100 torr and 0.05 torr helium. High pressure typically yields a 'smoke' of particles with sizes $<0.1 \mu\text{m}$, whereas the lower pressure yields polycrystalline material aggregated into more homogeneous thin films. As an alternative to the sublimation method, the powder was dissolved in benzene and drops of the solution were dried on the substrates.

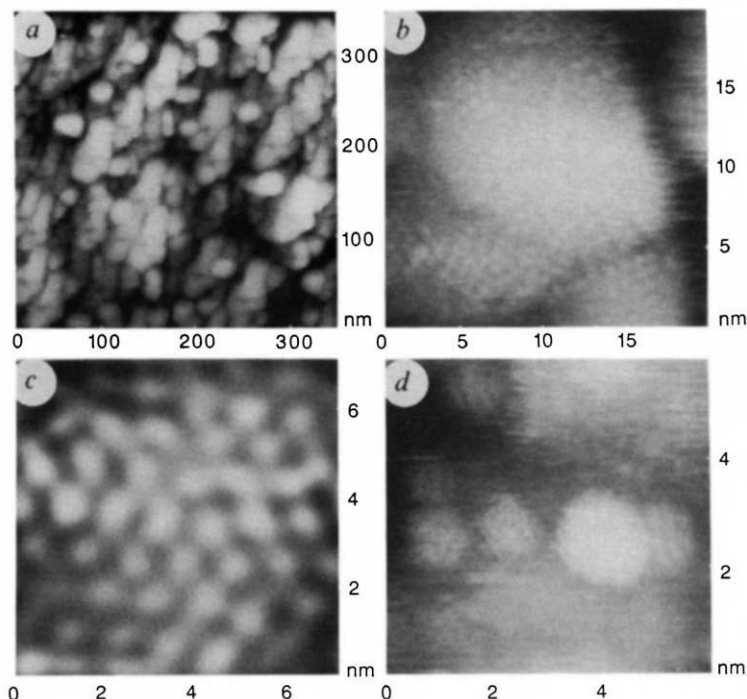
We used a NanoScope II scanning tunnelling microscope to image the samples. It was usually operated in the constant-current mode with the sample in air. Some images were recorded using the constant-height mode and some with the sample coated with mineral oil. Electrochemically etched tungsten tips were primarily used, but corroborative images were also recorded with mechanically formed platinum-iridium tips. The bias voltage applied between the tip and sample ranged from 10 to 260 mV, and was typically ~40 mV. The current setpoint range was 0.37–2.0 nA, with a typical value of 0.8 nA (ref. 6).

Figure 1 shows a sequence of images at increasingly higher magnifications, illustrating the character of fullerite. In Fig. 1a an image of a sample sublimed at 100 torr reveals that the C_{60}/C_{70} smoke particles consist of roughly spherical microcrystallites with diameters of ~20 nm. They can demonstrate high mobility between successive scans.

A view of the same sample at higher magnification (Fig. 1b) reveals the characteristic structure of the surface of an individual smoke microcrystallite. It consists of a highly defected array of spherical particles (structurally similar to a raspberry). The spacing of these smaller features is $1.0 \pm 0.1 \text{ nm}$, consistent with X-ray data of similarly prepared specimens⁵. The diameter of the 60-atom carbon cage is thought to be ~0.7 nm with the π -electron cloud extending outward from the molecule somewhat further. The apparent density of packing defects in images of this scale was quite variable, but generally a well ordered region extended only a few molecules. For the more highly disordered samples or for small, isolated clusters containing less than ten molecules the spacing of individual molecules was somewhat larger—up to 1.3 nm. The resolution in these images is not sufficient to distinguish between the spherical C_{60} and the possibly elongated C_{70} molecules.

Figure 1c shows a higher-magnification view of a sample sublimed at 0.05 torr. The small noise spikes in this image have been smoothed out by low-pass filtering to clarify the larger-scale

FIG. 1 STM images of the fullerite surface. *a*, $350 \times 350 \text{ nm}^2$ area showing the large-scale structure of the smoke particles, each roughly 20 nm in diameter. *b*, $20 \times 20 \text{ nm}^2$ image of a particle showing its structure of individual molecules. *c*, $7.2 \times 7.2 \text{ nm}^2$ image illustrating the packing arrangement of the accreted molecules. *d*, $6.0 \times 6.0 \text{ nm}^2$ image showing several 'normal' molecules in the presence of one larger feature, the size of which is consistent with the interpretation of it as a larger fullerene.



structure and to illustrate better the hexagonal packing arrangement of the individual molecules. X-ray diffraction⁵ has also shown the tendency for the molecules to arrange themselves into layers of this type.

Figure 1d is an interesting image of a sample dried from a benzene solution. This image was recorded with the sample under mineral oil, a commonly used technique of scanning tunnelling microscopy (STM) which often yields images with lower noise. There are several balls of the usual size in the company of a significantly larger ball, ~ 1.5 nm in diameter. This ball may be a larger fullerene such as C_{180} or C_{240} , although this is highly speculative⁷. This is the only such image we have found amid many thousands of fullerene molecules. The other significant aspect of the image in Fig. 1d is that some of the smaller balls show ridges roughly 0.3–0.4 nm apart, whereas others reveal no features. Ridges have been observed in many instances, both with and without the oil present. Although these features might be produced by the carbon cage underlying the π -electron cloud of the fullerenes, they may instead be simply a consequence of surface contamination of the molecules.

Figures 1a–c are images of the surface of bulk, solid fullerite.

The individual fullerene molecules that produce the images are in contact with other fullerene molecules, not interacting directly with the gold substrate. The mechanism by which the current flow enables these images to be recorded is not entirely clear. There are, however, many examples in the literature where nominally insulating species produce excellent images⁸. One possibility is that the presence of the tunnelling tip perturbs the molecules in such a way that suitable electronic states become available for occupation. Another possibility is that the necessary electronic states may arise from the alteration of molecular orbitals due to the proximity of neighbouring molecules when in the solid state.

These images corroborate other evidence for the existence of the fullerenes and provide information about the structure of their solid phase. Other applications of STM to the fullerenes will probably use the spectroscopic and manipulative potential of STM to study individual molecules in detail. One problem to be overcome is that of getting an individual molecule to stay in place under the influence of the probing tip. STM experiments at low temperature and in vacuum are the next steps. $\square$

Received 4 October; accepted 16 November 1990.

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ACKNOWLEDGEMENTS. J.L.W., J.E.C. and H.W.W. thank the U.S. Army Research Office for support.

Computer simulations of a water/oil interface in the presence of micelles

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AMPHIPHILIC molecules such as detergents or lipids, which contain a hydrophilic 'head' and a hydrophobic 'tail' are capable of forming a wide variety of complex structures, including micelles, vesicles, bilayers, monolayers and liquid crystalline structures. This property is essential in many biological processes and is exploited in industrial and domestic applications, but remains poorly understood at a molecular level. Here we present the results of computer simulations of a molecular model for an oil–water–surfactant system. Micelles form spontaneously in the water phase and a monolayer of surfactants forms at the oil/water interface. A depletion layer, containing only water, separates this monolayer from the micelles. The density profiles of the micelles and the water show pronounced oscillations, which result from packing constraints on the micelles near the interface. These oscillations in the water density profile furnish a possible explanation of the results of neutron reflectivity experiments on water–surfactant systems.

We performed molecular dynamics calculations on an oil–water–surfactant system. The model we used for this three-component system has two kinds of particle, which we label by the letters o and w respectively. An oil molecule consists of a single o particle and a water molecule consists of a single w particle. A surfactant molecule is a chain of two w particles followed by five o particles, each bound to its neighbour by a strong harmonic force. All particles interact by a Lennard–Jones potential $\phi(r) = 4\epsilon[(\sigma/r)^{12} - (\sigma/r)^6]$. The o–o and w–w inter-

action is truncated at 2.5σ . The o–w interaction is truncated at $2^{1/6}\sigma$ to make this interaction completely repulsive. As a result, the oil and water do not mix and form a stable liquid/liquid interface^{1,2}; the surfactant molecules are amphiphilic, one end is hydrophilic and dislikes oil, the other end likes oil and is hydrophobic^{2,3}. Monte Carlo simulations on a related model have been reported^{4,5}.

We performed our simulations at constant temperature ($T = 1.0\epsilon/k_B$) and volume on a network of 100 transputers using an efficient parallel-processing algorithm for molecular dynamics (K.E., P.A.J.H. and B.S., manuscript in preparation). The particles were initially placed on a face-centred-cubic lattice of size $30.4\sigma \times 30.4\sigma \times 60.8\sigma$. The density obtained was $\rho = 0.7\sigma^{-3}$, with 39,304 particles. Surfactants were introduced in a way that guarantees a spatially random distribution. All the remaining particles on one half of the lattice were water and on the other half oil. The surfactant concentration ranged from 1% to 3%. The system was equilibrated for at least 100,000 time steps ($\Delta t = 0.005(m\sigma/\epsilon)^{1/2}$), and then run for at least another 100,000 time steps. Because we use periodic boundary conditions, there are two interfaces and both are depicted in the figures.

Figure 1 shows density profiles for a surfactant concentration of 3%. The surfactants are preferentially adsorbed at the interface as a monolayer. The segment distribution reflects the expected orientational ordering (hydrophilic heads towards water). In the water phase, next to the monolayer, there is a depletion layer a few molecular diameters wide that contains almost no surfactant. The density profile of the water shows pronounced oscillations. The density profile of the oil phase also shows some oscillations but these are much less significant. Lower surfactant concentrations yielded similar results.

Figure 2 shows a typical instantaneous arrangement of the surfactants. In the water phase the formation of micelles has occurred. In the oil phase the surfactants also tend to cluster, but clearly defined reversed micelles cannot be discerned. In addition, this figure clearly shows the depletion layer.

We base our explanation of these observations on the fact that structure in simple liquids⁶, and even in some more complex systems such as liquid crystals⁷, is largely determined by hard-core interactions. Imagining the micelles to be hard spheres⁸

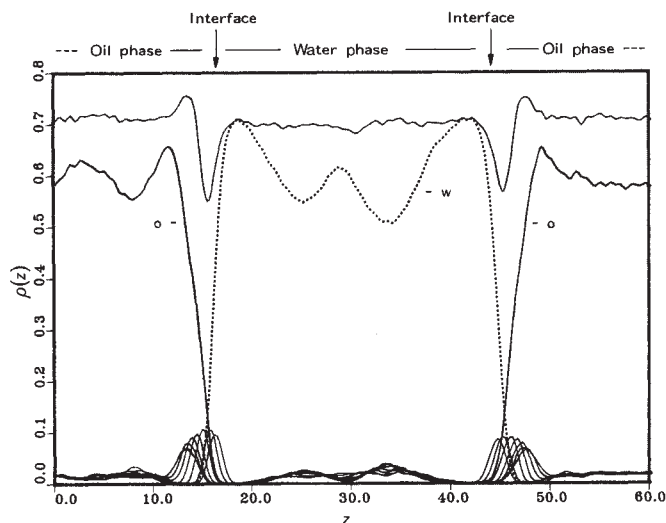


FIG. 1 Density profiles of the oil, water and surfactant particles. The green line gives the total density (oil, water and all surfactant segments). The dotted black line gives the density profile of the water particles, the solid black line of the oil particles. The blue lines give the density profiles of the two hydrophilic segments and the red lines the profiles of the five hydrophobic segments of the surfactant. The density is defined as the number of particles or segments per unit volume.

and the monolayer to be a wall, the system is equivalent to a hard-sphere fluid confined between parallel plates. In such a system, packing constraints cause characteristic oscillations in the density profile⁹. Similarly, packing constraints on the micelles will cause oscillations in their distribution. As the total density in the water phase is constant (see Fig. 1), the water molecules will fill the remaining space and thus the water density will oscillate, with a period of the order of the diameter of the micelle. In a real system there is only one interface, and the oscillations will be present in the neighbourhood of the interface.

If the interaction between the micelles and the monolayer was really the same as that between a hard sphere and a hard wall, then the first layer of micelles would be in contact with the monolayer and no depletion layer would be present. In our simulations, however, we observe a depletion layer, the width of which suggests a short-ranged repulsion between the micelles and the monolayer. Because the direct interactions between the heads of the surfactants are attractive in our model, this is somewhat surprising. This repulsive interaction is probably due to the water molecules between the micelles and the monolayer. Such an effective force between supramolecular structures caused by solvent molecules is often referred to as a solvation force¹⁰.

Whereas previous theoretical work has been concerned mainly with either micelles¹¹ or with a monolayer¹², our model involves both and can therefore be used to help interpret recent specular neutron reflection experiments^{13,14}. These experiments on the air/water interface of a solution containing ionic surfactants showed an 'unexpected' enhancement in the reflectivity of D₂O above the critical micelle concentration, which has not been explained satisfactorily. The oscillations of the density profile

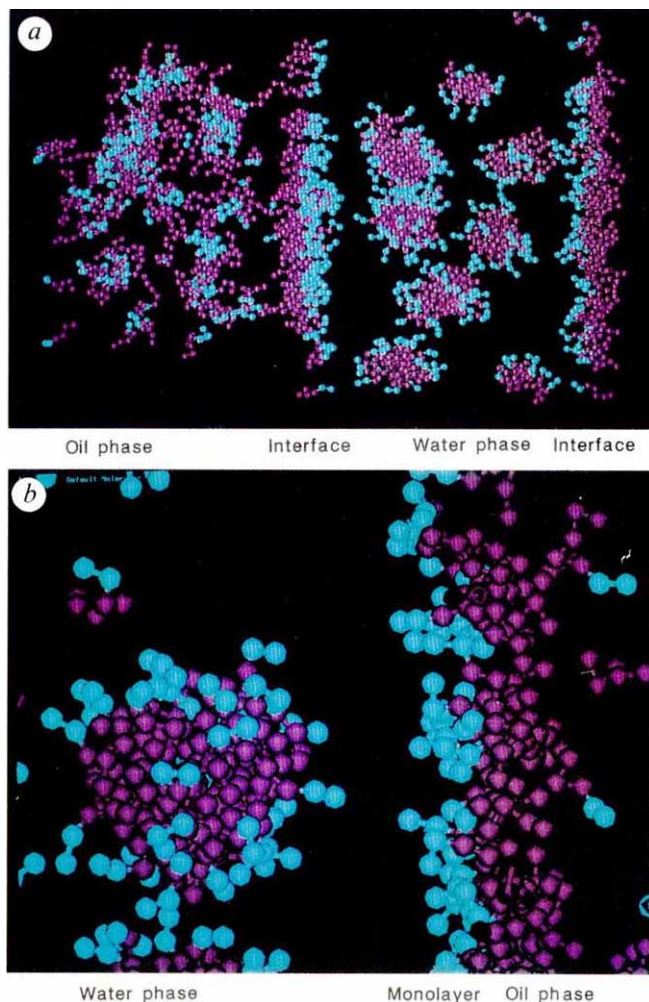


FIG. 2 *a*, Typical example of a configuration of surfactants in an oil-water system. The hydrophilic segments are blue and the hydrophobic segments purple. For clarity the positions of the oil and water particles are not drawn. *b*, Close-up of one of the micelles close to the monolayer.

of water as observed in our simulations, however, should give rise to just such an enhancement of the reflectivity. The location of the maximum, which corresponds to the period of the oscillations, depends on the size of the micelle, which in turn depends on the type of surfactant. Indeed, these dependencies have been observed in the reflectivity experiments¹⁴. Lee *et al.*¹⁴ have stated that an oscillating water density profile would explain the enhancement. They attribute the oscillations to a complicated layered structure of water and surfactant bilayers but our simulations indicate that these oscillations simply result from the packing constraints of micelles.

The depletion layer we have found may be of importance in understanding practical problems such as the rate of oil solubilization in micellar solutions. It is therefore of considerable practical importance to verify this layer experimentally. □

Received 13 August; accepted 30 October 1990.

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ACKNOWLEDGEMENTS. We thank W. de Jeu, R. Evans, D. Frenkel, B. Jérôme, G. Knowles, P. Lambooy, H. N. W. Lekkerkerker, R. Reijnhart, and I. Sziefler for comments and suggestions.

Future changes in stratospheric ozone and the role of heterogeneous chemistry

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HETEROGENEOUS chemical reactions on the surfaces of solid or liquid particles present in the lower stratosphere may be an important influence on the levels of ozone depletion resulting from increased concentrations of anthropogenic chlorine compounds in the atmosphere¹. Such processes, occurring on ice particles in polar stratospheric clouds, have been invoked to explain the formation of the springtime ozone hole over Antarctica^{2,3}. Sulphuric acid aerosols injected into the atmosphere following a volcanic eruption may also provide sites for heterogeneous chemistry leading to ozone depletion⁴. Here we present model calculations that assess the importance of heterogeneous processes in future ozone depletion, assuming that trace-gas concentrations follow the protocol agreed at the recent international convention in London. We find that, even if this protocol is adhered to, reactions on the surface of sulphuric acid aerosol particles could produce significant ozone depletion into the beginning of the next century, especially if a major volcanic eruption (similar to the El Chichón eruption of 1982⁴) takes place.

Stratospheric aerosols (solutions of sulphuric acid in water) are present in the lower stratosphere at all latitudes. The aerosol layer, discovered by Junge⁵, extends from about 10 to 30 km altitude; the aerosol concentration, and therefore the area available for heterogeneous chemistry, is dramatically increased after large volcanic eruptions (such as El Chichón). Laboratory work has shown that sulphuric acid aerosols can convert nitrogen oxides such as N_2O_5 into nitric acid (HNO_3), and inactive chlorine reservoirs ($ClONO_2$, HCl) into reactive (ozone-depleting) chlorine (Cl , ClO)^{6,7}. As the rates of these processes, as well as their dependence on aerosol composition and temperature are still uncertain, we have used a range of plausible rates and have performed sensitivity studies.

For the sake of clarity, we consider here only one scenario for future increase in trace-gas concentrations. This scenario, summarized in Table 1, accounts for a 95% phase-out of CFCs by the year 2000 (London agreement, 1990). The level of chlorine (1 part per 10^9 by volume (p.p.b.v.) in 1960 and 2.5 p.p.b.v. in 1980) will reach 4.5 p.p.b.v. in year 2010.

Figure 1a–c shows the calculated changes in the ozone column between 1960 and 2010 as a function of latitude and time of the year. The model⁸ used for these calculations is two-dimensional (latitude and altitude) and treats chemistry, radiative transfer and dynamics consistently in the stratosphere and mesosphere.

When heterogeneous processes are ignored (Fig. 1a), the calculated ozone depletions are modest: the reductions between 1960 and 2010 are typically 1% at the Equator; they vary with latitude, reaching 1% at 85°N and 1.5% at 85°S in late winter and early spring. The high-latitude ozone decrease is caused by downward transport of ozone-depleted air from the upper stratosphere during winter at high latitudes^{9,10}. Thus, when heterogeneous chemistry is neglected, the ozone depletion in the polar regions is primarily affected by the seasonal variation in the mean meridional circulation of the atmosphere. The positive changes seen at mid-latitude are caused by a cooling of the stratosphere resulting from the CO_2 increase (and consequently a reduced ozone destruction rate) and an enhanced

ozone production in the troposphere associated with the CH_4 increase. The global ozone reduction in this case is 0.22%.

When we included the effects of heterogeneous reactions on the surface of particles^{11,12} contained in polar stratospheric clouds (PSCs), the model showed dramatic ozone reductions in the polar lower stratosphere during the spring period and predicts a global ozone reduction of 3.6% between 1960 and 2010. Between 5 August and 5 October, the ozone density calculated at 85°S is reduced by 3% at 28 km, 68% at 25 km, 71% at 20 km, 59% at 15 km and 10% at 10 km. The October mean ozone column at the same latitude is reduced from 315 dobson units in 1960 to 295 in 1970, 245 in 1980, and 185 in 1990.

Finally, when we included the effects of stratospheric aerosols, the model predicted further ozone depletion. The composition

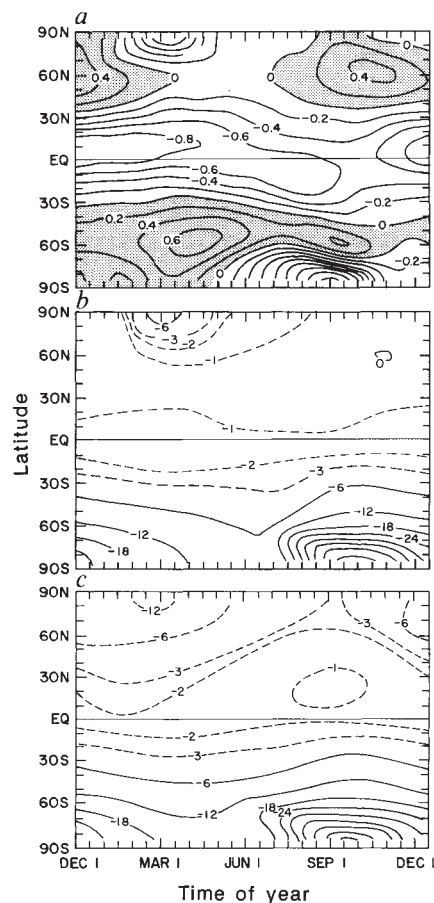


FIG. 1 Ozone column changes (per cent) as a function of latitude and season calculated between 1960 and 2010 when a, only homogeneous chemistry processes are taken into account; b, heterogeneous processes occurring on the PSCs in the polar regions are introduced; c, heterogeneous processes on both PSCs and sulphate aerosols are considered (reaction probabilities $\gamma_1 = 2.6 \times 10^{-3}$ and $\gamma_2 = 0.14$). The two-dimensional model used here extends from pole to pole and from the surface to 85 km altitude. The chemical scheme includes 56 species belonging to the O_x , HO_x , ClO_x , BrO_x and SO_x families as well as methane and its oxidation products. Chemistry, radiative transfer and dynamics are treated interactively, above 10 km altitude, so that the most important feedbacks (such as ozone–temperature) are taken into account. When we included the chemical effects of the PSCs, we assumed that as the temperature of the lower stratosphere decreases below 199 K in the two polar regions, the two chlorine reservoirs HCl and $ClONO_2$ are converted with a time constant of 15 days into a more reactive form of chlorine ($ClO_x = Cl_2 + ClO + OClO + Cl_2O_2$) and that N_2O_5 is converted into HNO_3 . When type II PSCs are formed below ~ 194 K, we assumed that the lower stratosphere is denitrified by gravitational sedimentation of the relatively large ice particles involved. The parameterization of the heterogeneous chemical processes on the surface of sulphate aerosol particles is described in the text. For each simulated case, the model was run to steady-state conditions with fixed boundary conditions.

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TABLE 1 Concentrations used in the model simulations

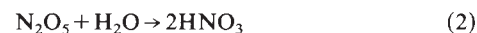
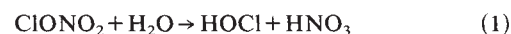
	1960	1970	1980	1990	2010
CO ₂ (p.p.m.v.)	316	327	339	352	381
N ₂ O (p.p.b.v.)	289	295	302	310	326
CH ₄ (p.p.b.v.)	1,255	1,375	1,525	1,670	1,975
CH ₃ Cl (p.p.t.v.)	600	600	600	600	600
CFC-11 (p.p.t.v.)	11	60	173	275	286
CFC-12 (p.p.t.v.)	33	121	297	468	544
CFC-113 (p.p.t.v.)	0.2	2.3	15.3	51	72
CFC-114 (p.p.t.v.)	0.2	1.4	3.8	7	10
CFC-115 (p.p.t.v.)	0	0.2	2.1	5	6
HCFC-22 (p.p.t.v.)	1	10	54	111	567
halon-1211 (p.p.t.v.)	0	0.1	0.5	1.8	1
halon-1301 (p.p.t.v.)	0	0.1	0.6	3.2	4.8
CCl ₄ (p.p.t.v.)	75	85	95	105	120
CH ₃ CCl ₃ (p.p.t.v.)	5	55	105	150	230
Total Cl _x (p.p.b.v.)	1.01	1.55	2.52	3.5	4.5

We have assumed a 95% phase-out of CFCs by the year 2000, with 50% of this reduction (in mass) being added to the base emission of HCFC-22 (which acts as a surrogate for all CFC replacement products, except the halons). CCl₄ was assumed to grow by 1 p.p.t.v. per year from 1960 to 1985 and 4 p.p.t.v. per year afterwards. CFC-11, CFC-12, CFC-113, CFC-114, CFC-115, C₂F₅Cl; HCFC-22, CHF₂Cl; halon-1211, CF₂ClBr; halon-1301, CF₃Br.

of the aerosols observed in the lower stratosphere is consistent with a sulphuric acid/water mixture varying from ~60 to 80% sulphuric acid¹³. The rate at which atmospheric molecules react on the surface of aerosol particles is proportional to the mean molecular velocity of the molecule (which is a function of its mass and temperature), the surface area of aerosols available per unit of volume, and the probability that a collision results in a chemical reaction⁴. The surface area available depends on the aerosol load and the size of the particles present in the atmosphere. If no major volcanic eruption has perturbed the atmosphere for several (>10) years (background atmosphere), the aerosol surface area in the lower stratosphere is typically $0.5 \mu\text{m}^2 \text{cm}^{-3}$. After a large volcanic eruption, it can reach values

of $10\text{--}50 \mu\text{m}^2 \text{cm}^{-3}$. Two cases are therefore distinguished: in the first of them (background atmosphere), we adopted the vertical profile of aerosol surface area observed in September 1979 at Laramie, Wyoming⁴. Values at other latitudes are obtained by translating, parallel to the tropopause, the Laramie profile. In the second case (volcanic period), we adopted a two-dimensional distribution representative of the situation at the end of 1982 after the eruption of El Chichón, based on balloon measurements at Laramie¹⁴ and on aircraft observations of integrated aerosol backscattering¹⁵ from 46°N to 46°S . The largest aerosol load was located between 40°S and 60°N , with the highest surface area being $\sim 35 \mu\text{m}^2 \text{cm}^{-3}$ (at 24 km altitude near the Equator). At the time the profile was measured, only a limited amount of aerosol had been transported into the Southern Hemisphere and the region where aerosol surface area was larger than $15 \mu\text{m}^2 \text{cm}^{-3}$ extended from 10°S to 50°N .

Reaction probabilities, were measured in the laboratory. Their values depend strongly on the acid content of the aerosol, which in turn depends on the ambient temperature and the relative humidity. In the model simulation, we assumed that ClONO₂ and N₂O₅ were converted heterogeneously by^{6,7}



Data concerning the probabilities of these reactions are few and

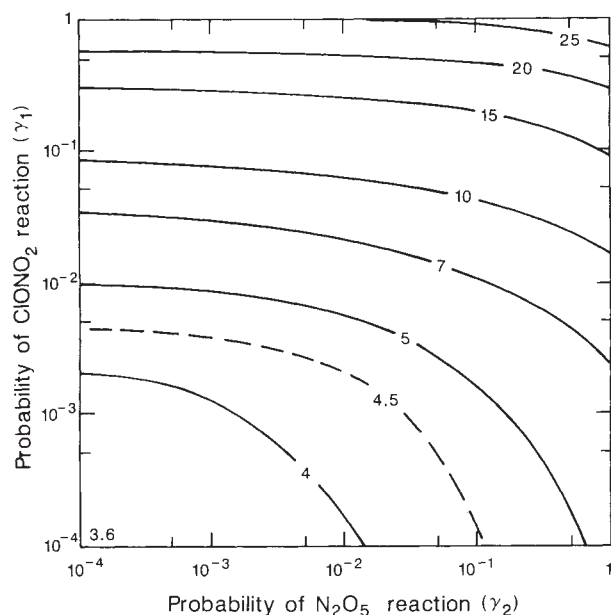


FIG. 2 Global ozone decrease (per cent) between 1960 and 2010, as a function of the heterogeneous reaction probabilities γ_1 and γ_2 (background aerosol level; no volcanic perturbation). The depletion is 3.6% when the effect of sulphate aerosols is ignored. The dashed line gives the combination of the γ_1 and γ_2 values for which the reduction in global ozone is enhanced by ~1%, assuming a background aerosol load.

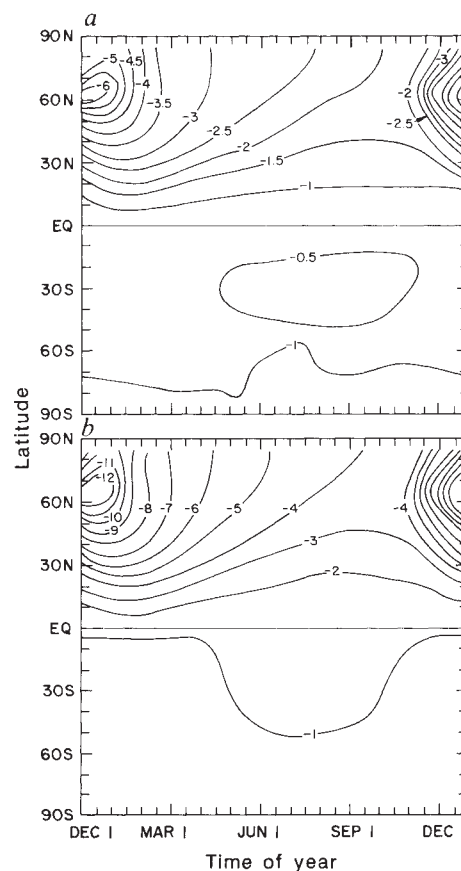


FIG. 3 Relative change (per cent) of the ozone column abundance as a function of latitude and season calculated (at steady state) for an aerosol load changing from a background to a post-volcanic situation. The volcanic eruption is assumed to be similar to that of El Chichón in 1982. The reaction probabilities are $\gamma_1 = 2.6 \times 10^{-3}$ and $\gamma_2 = 0.14$ (see text). In a and b, the chlorine level in the atmosphere corresponds to that of 1982 and 2010, respectively. The largest ozone response to a volcanic perturbation occurs at high latitudes in winter. The maximum ozone drop is predicted to be twice as large in 2010 as it was in 1982.

the values are uncertain by at least an order of magnitude. For the probability of reaction (1), γ_1 , Tolbert *et al.*⁶ report values of 3×10^{-4} and 2.6×10^{-3} for 75 and 65wt% of acid, respectively. According to laboratory work using non-dispersive sulphuric acid aerosol¹⁶, the probability of reaction (2), γ_2 , could be as high as 0.09 (293 K) to 0.14 (274 K) and relatively independent of the relative humidity (1–10%). The value of γ_2 is not known for the solid or the supercooled sulphuric acid aerosols which would exist at stratospheric temperatures.

Figure 1c shows the change in the ozone column between 1960 and 2010 with the added influence of the background aerosol layer. For this calculation we used $\gamma_1 = 2.6 \times 10^{-3}$ and $\gamma_2 = 0.14$ (ref. 17). When comparing Fig. 1c to Fig. 1b, the addition of the background aerosol modifies the predicted ozone depletion, which is now 5.7%. For potentially larger values of the reaction probabilities, however, the contribution of aerosol particles becomes more significant; as shown by Fig. 2, the effect of aerosols at their background level on the global ozone budget becomes noticeable (additional ozone depletion of $\geq 1\%$) for γ_1 and γ_2 becoming larger than $\sim 3 \times 10^{-3}$ and 0.1, respectively.

For an aerosol load corresponding to a post-volcanic situation, the sensitivity of ozone to chlorine is substantially enhanced. Figure 3a, b shows the change in the ozone column resulting from an increase in aerosol surface area from the background to the post-volcanic situation, for unchanged concentrations of trace gases at their level of 1982 and 2010, respectively (see Table 1). The values of γ_1 and γ_2 are the same as those used for the model simulation shown in Fig. 1c. Figure 3b suggests that, even with reduced emissions (nearly total phase-out) of CFCs in the future, a large volcanic eruption in year 2010 or so, could deplete ozone significantly at all latitudes: the depletion in global ozone calculated for 2010 is 2.3% larger than for the background aerosol case (total ozone depletion compared to 1960 including PSC and volcanic effects is 8%). The magnitude of the ozone change depends dramatically on the reaction probabilities used and laboratory investigations of these values as a function of temperature and aerosol composition are urgently needed. This last model calculation shows, however, that heterogeneous chemistry should affect the predictions of ozone change, especially if large quantities of aerosol particles are injected into the lower stratosphere (provided that the laboratory values derived for γ_1 and γ_2 are representative of sulphuric acid aerosols at stratospheric temperatures). Ozone depletion has been observed after the eruption of El Chichón^{4,18–20}. Since 1982, the level of chlorine in the atmosphere has increased by $\sim 30\%$ and it is expected to increase further by 2010. According to the model, the ozone sensitivity to volcanic eruptions should be twice as large in 2010 as it was in the early 1980s. Thus, despite limitations in the emissions of CFCs, noticeable changes of anthropogenic origin could be observed in the next 20 years. □

Received 30 July; accepted 25 October 1990.

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ACKNOWLEDGEMENTS. We thank J. Calvert, A. Fried, R. Garcia and M. Mozurkewich for comments. C. G. is supported by a grant from the European Space Agency. This research was supported in part by the STEP Program of the Commission of the European Communities. The National Center for Atmospheric Research is sponsored by the NSF.

Mechanism of the γ - β phase transformation of Mg_2SiO_4 at high temperature and pressure

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THE transformation of $\text{Mg}_{1.8}\text{Fe}_{0.2}\text{SiO}_4$ olivine first to the β -phase (modified spinel) and then to the γ -spinel phase occurs with increasing depth in the Earth's mantle as a result of the increasing pressure^{1,2}. The mechanisms of these two transformations have an important influence on mantle rheology and may also be related to the origin of deep-focus earthquakes^{3–8}. We report here the results of experiments on the phase transformation of Mg_2SiO_4 olivine at 15 GPa pressure in a multi-anvil cell. At this pressure and a temperature of 900 °C, early formed metastable γ -spinel transforms partially to the β -phase. The observed microstructures, which are similar to those in shocked meteorites^{9–11}, show that the γ -to- β transformation can occur either by diffusion-controlled growth or by a martensitic (shearing) mechanism, depending on how far the pressure-temperature conditions deviate from their values at phase equilibrium. Our results suggest that the diffusion-controlled mechanism is most likely to operate at the β/γ phase boundary in the mantle, but a martensitic β -to- γ transformation might occur in subduction zones and could reduce the shear strength of the subducting slab.

We performed high-pressure experiments using a 1,000-ton uniaxial split-sphere apparatus^{12,13}. The sample assembly consisted of an MgO octahedron, with an edge length of 10 mm, containing a cylindrical Pt heater^{14,15}. The starting material for the experiments was Mg_2SiO_4 olivine (forsterite) powder which was packed into the full length of the Pt heater. The temperature was measured at the centre of the sample using a Pt-Pt13%Rh thermocouple. Because the ends of the sample were in contact with the tungsten carbide anvils and therefore at relatively low temperature, there was a significant temperature gradient along the axis of the sample.

Experiments were initiated by hot-pressing the forsterite powder in the olivine stability field at 12 GPa and 1,200 °C (at the thermocouple) for 1 h (Fig. 1). Then, by reducing the temperature to 900 °C and further increasing the pressure to 15 GPa, the hot-pressed forsterite was partially transformed to its high-pressure polymorphs in two experiments, for times of 1 h and 5 h. We studied the products of the experiments by transmission electron microscopy (TEM) as well as X-ray diffraction and light optical microscopy¹⁵.

In the 1-h experiment, the forsterite within 300 μm of the thermocouple partially transformed to its high-pressure polymorphs with a grain size $< 1 \mu\text{m}$. Although β -phase was the predominant product, $\sim 10\%$ of the new grains consisted of γ -spinel. The spinel grains contain a high density of faults parallel to $\{110\}$ which can be easily detected at low magnification in the TEM. High-resolution images (Fig. 2a) show that the faults consist of lamellae of β -phase which typically vary from one to six unit cells in width. The high-resolution images of these β -phase lamellae together with selected-area

electron diffraction patterns of the spinel show that the two phases are orientated with $[001]_{\beta} \parallel [001]_{\gamma}$ and $[010]_{\beta} \parallel [110]_{\gamma}$. Some grains of β -phase also contain numerous faults with the spinel structure. The spinel lamellae are typically either one, two or three unit cells in width (Fig. 2b) and have the same orientation relationship to β -phase as in the grains consisting predominantly of spinel.

After reaction for 5 h, the forsterite starting material was completely transformed to fine-grained β -phase (grain size $\sim 1 \mu\text{m}$) up to 1.3 mm from the thermocouple (Fig. 3a). At the ends of the sample, where temperatures were low, transformation failed to occur for kinetic reasons. In contrast to the result of the 1-h experiment, spinel is absent as discrete grains close to the thermocouple after 5 h, and stacking faults in β -phase, which have the spinel structure, are rare. We did find spinel associated with β -phase close to the boundary with the relict metastable forsterite ($\sim 1.3 \text{ mm}$ from the thermocouple). In this region, although grains of β -phase predominate, composite grains consisting of β -phase and spinel separated by irregular phase boundaries also occur (Fig. 3b). The two phases are orientated with $[001]_{\beta} \parallel [001]_{\gamma}$ and $[010]_{\beta} \parallel [110]_{\gamma}$, as in the lamellar intergrowths described above.

The phase diagram (Fig. 1), together with the predominance of β -phase as a reaction product in both experiments, shows that transformation occurred entirely in the β -phase stability field. The presence of limited amounts of spinel, especially after 1 h, is most likely due to the metastable nucleation and growth of this phase. Such behaviour is consistent with the Ostwald step rule provided the P - T conditions of reaction lie at a pressure in excess of that of the metastable α/γ equilibrium boundary¹⁵. TEM observations show that both phases formed from forsterite by a mechanism involving incoherent nucleation and diffusion-controlled growth¹⁵.

The lamellar intergrowths of β -phase and spinel, which are present within $300 \mu\text{m}$ of the thermocouple after 1 h (Fig. 2), have two possible origins. The intergrowths could be original growth features (stacking faults) in which lamellae of spinel result from mistakes in the stacking sequence during the growth

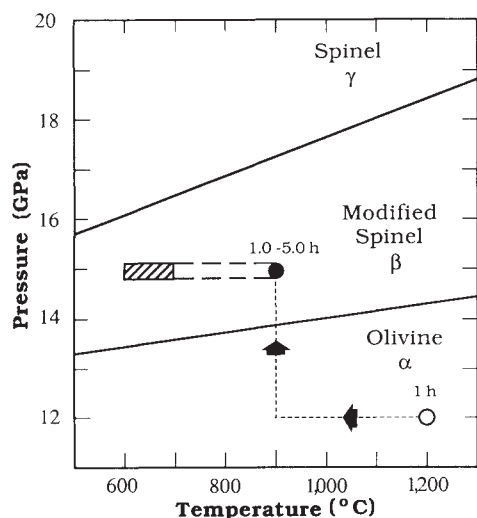


FIG. 1 Mg_2SiO_4 phase diagram showing the stability fields of olivine (α), modified spinel (β) and spinel (γ) (after Akoagi *et al.*²⁵). The P - T path followed by the central part of the sample to reach 900°C and 15 GPa (solid circle) from the conditions of hot pressing (open circle) is shown by the dashed line with arrows. The temperature variation across the part of the sample that has reacted for 5 h at 15 GPa is shown by the broken lines. Evidence for the reaction of spinel to β -phase by a diffusion-controlled growth mechanism was found at the low-temperature end of this gradient (cross hatched), whereas evidence for the martensitic mechanism was found close to the thermocouple (solid circle) where there was a greater deviation of P - T conditions from equilibrium.

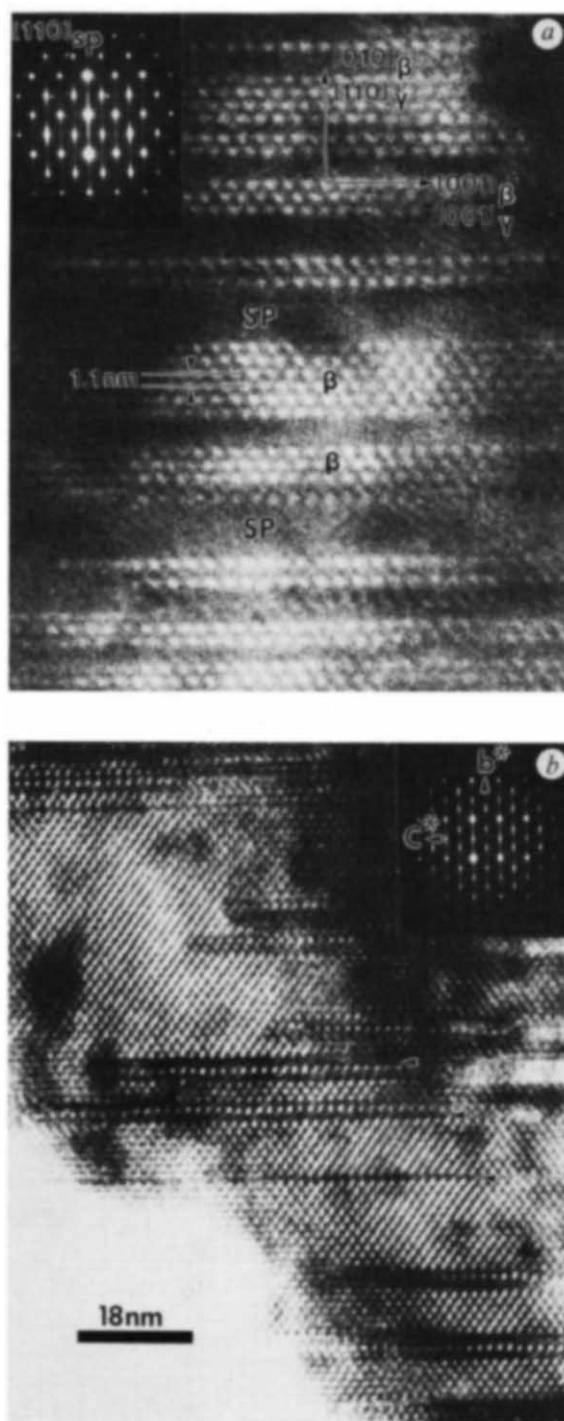


FIG. 2 High-resolution transmission electron micrographs of intergrown spinel (SP) and β -phase from close to the thermocouple in the sample reacted for 1 h. Because the high-pressure polymorphs of Mg_2SiO_4 , particularly β -phase, were damaged rapidly by the electron beam, it was only possible to obtain such images at relatively low magnifications ($<150,000\times$). a, Region of a grain of spinel that locally contains a high proportion (about 50%) of lamellae of β -phase on (110). The inset electron diffraction pattern, taken using a $20\text{-}\mu\text{m}$ diffraction aperture, is dominated by reflections from the spinel. The strong streaking parallel to $[110]_{\gamma}$ is caused by the disordered intergrowth of thin lamellae of spinel and β -phase. These lamellae are not sufficiently thick to diffract coherently. b, High-resolution image of a grain of β -phase imaged with the electron beam approximately parallel to the a axis. The β -phase contains abundant faults on (010) which have the spinel structure (arrows). The spinel lamellae are 1-3 unit cells wide and in some cases can be seen to terminate in the β -phase. The inset diffraction pattern ($[100]$ zone axis) shows strong streaking parallel to b^* owing to the thin lamellae of spinel.

of β -phase^{10,11,16}. Alternatively, they could result from the partial martensitic transformation of early formed metastable spinel to the stable β -phase^{9,11}. This martensitic transformation involves the propagation of $1/4[1\bar{1}2](110)$ partial dislocations on every alternate (110) plane of the spinel and transforms a layer of spinel two unit cells wide into a layer of β -phase one unit cell wide¹¹. The β -phase layer formed by this process is orientated with respect to the spinel lattice such that $[001]_{\beta} \parallel [001]_{\gamma}$ and $[010]_{\beta} \parallel [110]_{\gamma}$, as we observe.

The complete absence of spinel within 300 μm of the thermocouple after 5 h at 15 GPa and 900 °C shows that the early formed metastable spinel progressively transformed to the stable β -phase, with complete transformation occurring in <5 h at 900 °C. There is no evidence from the 1-h experiment that this transformation involved diffusion-controlled growth at 900 °C. We therefore conclude that the lamellar intergrowths observed after 1 h resulted mainly from the partial martensitic transformation of spinel to β -phase. The presence of relict forsterite crystals with dislocation densities $<10^8 \text{ cm}^{-2}$ indicate a differential stress $<0.1 \text{ GPa}$ during the martensitic transformation^{15,17}. This is low compared with the differential stress of $\sim 2 \text{ GPa}$ required to partially convert olivine to spinel by a martensitic mechanism¹⁸.

After reaction for 5 h relict spinel is only found in a region of the sample where temperatures were relatively low and P - T conditions were relatively close to equilibrium (Fig. 1), so that the rate of transformation to β -phase was slow. The composite grains, in which β -phase and spinel are separated by an irregular boundary (Fig. 3b), provide evidence for the transformation of spinel to β -phase by a mechanism in which the interphase boundary sweeps through the crystal, just as was found for growth of martensitic nuclei of spinel in $\alpha\text{-Mg}_2\text{GeO}_4$ (ref. 18).

This process must involve diffusion-controlled growth of β -phase and is comparable to a massive transformation¹⁹. The orientation relationship between the two polymorphs shows that nucleation occurred by the martensitic mechanism. It has been proposed that a similar two-stage transformation mechanism operated during the post-shock pressure release in the Peace River meteorite⁹.

These results show that two distinct growth mechanisms can operate during the γ -to- β transformation, depending on the P - T conditions. In our experiments, the martensitic mechanism operated at the P - T conditions that deviated most from the γ - β equilibrium boundary, whereas diffusion-controlled growth occurred at conditions relatively close to equilibrium (Fig. 1). This is consistent with studies of metallic systems, which show that martensitic transformations require a large chemical driving force (free energy of reaction) compared with diffusion-controlled or massive transformations which, because they are thermally activated, can operate at a low chemical driving force²⁰.

The occurrence of martensitic nucleation but diffusion-controlled growth in the low-temperature region of the sample reacted for 5 h shows that, because of the relatively low driving force, only very limited martensitic transformation could occur at these conditions. The absence of incoherent nucleation is presumably due to both the low temperature and low driving force. A recent study of γ -to- β transformation in Co_2SiO_4 , however, shows that both incoherent nucleation and diffusion-controlled growth occur fairly close to equilibrium at temperatures much higher than used here²¹.

Although transformation from spinel to β -phase by a martensitic mechanism has previously been recognized in shocked

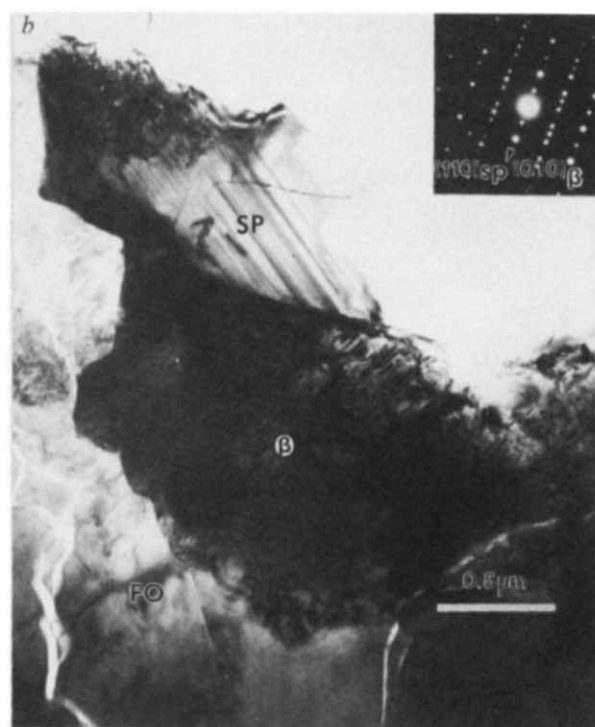
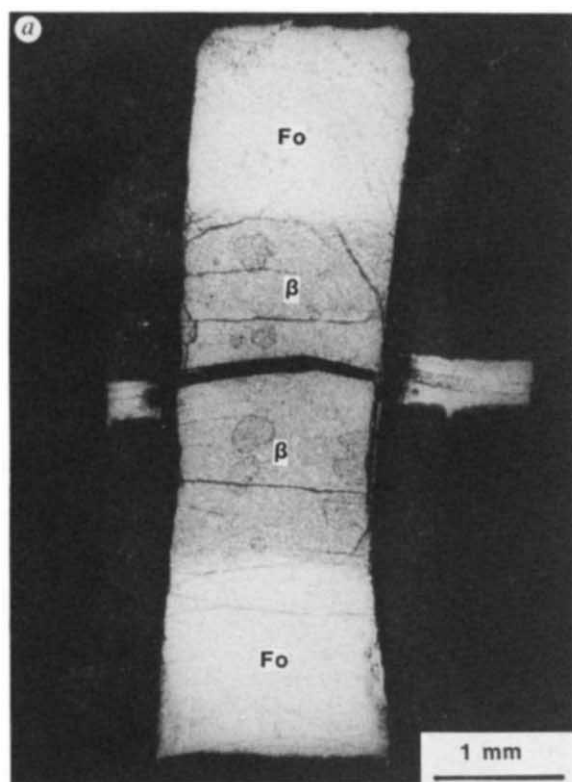


FIG. 3 *a*, Optical micrograph of the sample reacted for 5 h showing the central region consisting of β -phase and containing the thermocouple (black), and metastable forsterite (Fo) at the ends of the sample where temperatures were too low for transformation to occur. The temperature at the boundary between metastable forsterite and β -phase is estimated to have been 600–700 °C from kinetic data for the breakdown of olivine at high pressure²⁶ (see Fig. 1). *b*, Transmission electron micrograph of a composite grain of β -phase and spinel from close to the boundary between β -phase and

metastable forsterite shown in *a*. Relict forsterite (Fo) is also present. The planar structures in the spinel are $[110]$ cation stacking faults of a type that has been observed in germanate spinel¹⁶. The inset electron diffraction pattern taken across the β -phase/spinel phase boundary shows that the two phases are orientated with $[010]_{\beta} \parallel [110]_{\gamma}$. The complex mottled microstructure in the β -phase is caused by the presence of abundant small antiphase domains.

meteorites⁹⁻¹¹, the transformation conditions (P , T and differential stress) are unknown. Our work provides evidence that this mechanism could occur under mantle conditions. The transformation mechanism is potentially of considerable importance for mantle rheology, and therefore mantle dynamics, because if transformation occurs between β -phase and spinel by the martensitic mechanism, the shear strength should be greatly reduced during the transformation^{3,10}. Transformation plasticity may also occur during a diffusion-controlled transformation, but the mechanism and magnitude of the effect will be different^{4,22}. In either case, a zone of decoupling at the phase boundary in the mantle is a possibility⁶ and the rheology and dynamics of mantle plumes could also be affected as rising material crosses from the stability field of spinel to that of the β -phase. The details of the dependence of the different nucleation and growth mechanisms on P , T and differential stress have yet to be investigated. Our results together with those of Remsburg and Liebermann²¹ suggest, however, that at the β/γ phase boundary, where temperatures are $\sim 1,500^\circ\text{C}$ ²³, transformation will occur close to equilibrium by the diffusion-controlled mechanism.

By analogy with our results, the mechanism of the β -to- γ transformation is also likely to vary with pressure and temperature. Madon and Poirier¹¹ have shown that the energetics of a martensitic β -to- γ transformation are similar to those of the reverse transformation studied here. The martensitic β -to- γ transformation would involve the propagation of $1/2[101](010)$ partial dislocations on every alternate (010) plane of β -phase^{10,11} and would also be likely to result in a large reduction in shear strength. This mechanism is most likely to operate in subducting slabs where low temperatures inhibit a diffusion-controlled mechanism. Such martensitic transformation could have an important effect on the rheology of the slab. In addition, if the martensitic mechanism operates in subducting slabs under high differential stress, the transformation could occur very rapidly. Rapid transformation with an associated reduction in strength could result in shear instabilities and thus in deep-focus earthquakes. The occurrence of acoustic emissions during rapid martensitic transformations in Si and Ge at high pressure in the diamond-anvil cell has also led to the proposal that martensitic transformations could be the cause of some deep-focus earthquakes in subducting slabs²⁴. $\square$

Received 20 July; accepted 24 October 1990.

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ACKNOWLEDGEMENTS. We thank E. Ito for assistance with the high-pressure experiments, which were performed at Okayama University. Electron microscopy was carried out at the Electron Microbeam Analysis Facility, Department of Geology and Institute of Meteoritics, University of New Mexico. We thank H. W. Green for a review, and S. H. Kirby, S. Seifert and S. L. Webb for comments. This work was partially funded by NERC(D.C.R.) and NASA (K. Keil).

Evidence for reflectors in the lower continental crust before rifting in the Valencia trough

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THE past decade has seen a rapid advance in our understanding of the deep structure of the continental crust, especially in Europe¹⁻³, Australia⁴ and North America⁵, where seismic profiling has revealed a highly reflective layer in the lower crust. On many profiles, the reflective layer is associated with regions of extension^{6,7}, suggesting that the reflectors originate by some form of ductile flow or magmatic intrusion during rifting^{8,9}. To test this hypothesis, we have examined geophysical and geological data from the Valencia trough, a rift basin¹⁰⁻¹² of Late Oligocene/Early Miocene age. From a comparison of profiles in different parts of the trough, and independent constraints on the initial crustal thickness, we argue that the reflective layer in this basin was probably already in the crust before rifting, and that a mid-Tertiary extensional event is not required to explain it.

In November 1988, a two-ship (R/V *Robert D. Conrad* and N/O *Jean Charcot*) multichannel seismic reflection and refraction experiment was carried out in the Valencia Trough off the northeast coast of Iberia. The data include a 110-km-long common depth point (CDP) line 819 on the shelf, a 30-km-long CDP line 815 on the slope and a 100-km-long expanding spread profile (ESP 6) that crosses both the shelf and slope (Fig. 1). CDP line 819 and ESP 6 were shot normal to a steep gradient of the Bouguer gravity anomaly which marks¹³ the transition from the thick crust of Iberia to thin crust beneath the trough. Both ships were equipped with a 96-channel 2.4-km-long streamer, satellite navigation, and precision gravity and depth recorders. The 10-airgun tuned array of the *Conrad* was used as the source for both the CDP and ESP experiments.

Figure 2 shows a migrated 96-fold stack along line 819 together with results of velocity modelling at ESP 6. We used standard processing techniques¹⁴ and stacking velocities based on sonic velocities from nearby commercial wells. The well data¹⁵ indicates that the sedimentary layer is 3–4 km thick and of Early Miocene to Recent age. Underlying the sediments is a strong continuous reflector (2.2 s) which marks the surface of an eroded Mesozoic carbonate platform. Below this reflector, there is a thick, acoustically transparent layer with few internal reflectors. At a two-way time (TWT) of ~ 6.2 s, there is an abrupt development of subhorizontal lower crustal reflectors, which appear continuous on horizontal length scales of up to 15 km. Along parts of the profile, the reflective crust is characterized by lozenge-shaped transparent zones and concave-up cross-cutting reflectors. The reflectors end sharply at ~ 8 s where they pass into a strong continuous reflector which we interpret as the 'reflection' Moho. The average p-wave (compressional) velocities V_p of the upper and lower crustal layers at ESP 6 (Fig. 2) are 6.1 and 6.7 km s⁻¹, respectively, indicating a total crustal thickness (excluding sediments) along the line of 18–19 km.

Similar lower crustal reflectors have already been identified elsewhere in Europe^{1-3,16-20}. Typically²¹, these reflectors occur

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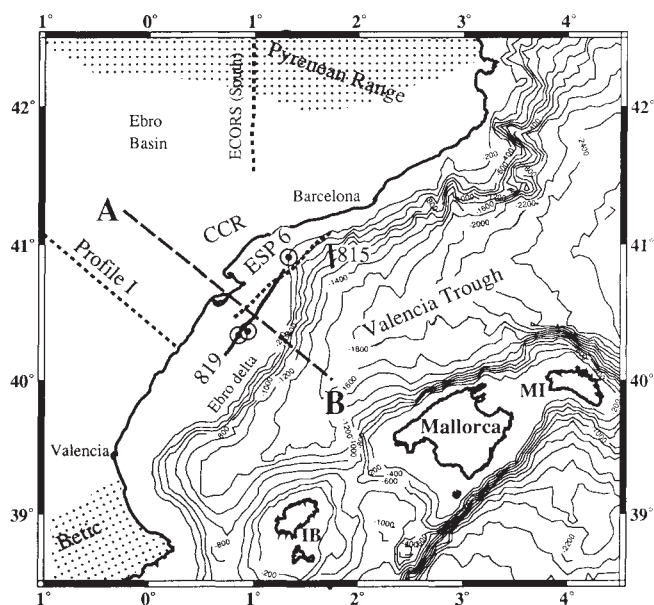


FIG. 1 Location map of the Valencia trough and vicinity. Topography³⁹ at 200-m interval. The solid lines show the location of CDP lines 819 and 815. The heavy dashed line shows the cross-section AB based on seismic and gravity modelling, the lighter dashed lines show the seismic refraction profiles I²² and ESP 6, and the seismic reflection profile ECORS Pyrenees South line³⁷. The filled circles show the wells used in Fig. 4. The wells from north to south are: 466, 419 and 299. CCR, Catalan Coastal Ranges; MI, Menorca; IB, Ibiza.

at a TWT of 5–7 s (15–21 km depth), have high average V_p (6.6–7.8 km s⁻¹) and are especially well developed in regions dominated by extensional tectonics.

Previous seismic refraction studies²² indicate crust 30–35 km thick beneath Iberia which, together with our results (Fig. 2), suggests at least 9 km of crustal thinning towards the Valencia trough. Were the reflectors generated by the same extensional event that formed the trough, were they added to it later, or were they already in the crust before extension? If the deep layers were produced in some way by extension, we would expect, following the arguments of Peddy *et al.*²³, the intensity of reflectors to increase towards the trough axis because this would be a region of thinner, more stretched crust.

Figure 3 shows part of line 815, which was shot at depths of ~800 m. This line does not show a highly reflective lower crust. Instead, there is a single continuous reflector at a TWT of ~8 s which is similar to the 'reflection' Moho on line 819 and to reflectors identified elsewhere²⁴ in the trough. We do not believe that the absence of reflectors in Fig. 3 is caused by a decrease in signal-to-noise ratio as the depth increases, scattering owing to the relief of the Messinian erosional discordance, or by lack of multiple suppression, although these factors may contribute. Rather, their absence suggests that the reflectors beneath the shelf were either pre-existing and a subsequent extensional event reduced their thickness beneath the trough or that they were added later by magmatism, which preferentially underplated²⁵ or intruded the shelf rather than the trough.

The crustal thinning at the time of rifting has been estimated by backstripping²⁶ the Late Oligocene/Early Miocene to Recent sedimentary section at three commercial wells drilled along line 819. The total tectonic subsidence (TTS)²⁷, which is in the range 1.4–1.6 km, could be explained by an instantaneous-stretching model²⁸ with an age of rifting of 25 Myr and a stretching factor of $\beta = 1.40$ –1.55 but, a better fit to the subsidence history was obtained (Fig. 4) using a finite-stretching model^{29,30} with a similar β and a rifting duration of 8–24 Myr. Evidence that rifting in the Valencia Trough may have been a protracted event is seen in the occurrence of calc-alkaline volcanic rocks of early

Miocene to middle Pleistocene age in Deep Sea Drilling Project sites in the trough³¹ and outcrop on the Columbretes Islands³². A stretching factor of 1.40–1.55 implies a crustal thickness of 20–22 km along line 819, assuming an initial crustal thickness of 31.2 km.

Figure 5 shows a crustal cross-section (profile AB) from the Ebro basin to the trough axis which is constrained by seismic, gravity anomaly and well data. The hatched lines (lower panel) show prominent discontinuities in V_p along seismic refraction profiles I²² and ESP 6 (Fig. 2). The mantle velocity of 7.8 km s⁻¹ is low when compared to velocities²² beneath Iberia but, is similar to velocities beneath active 'continental' rifts such as

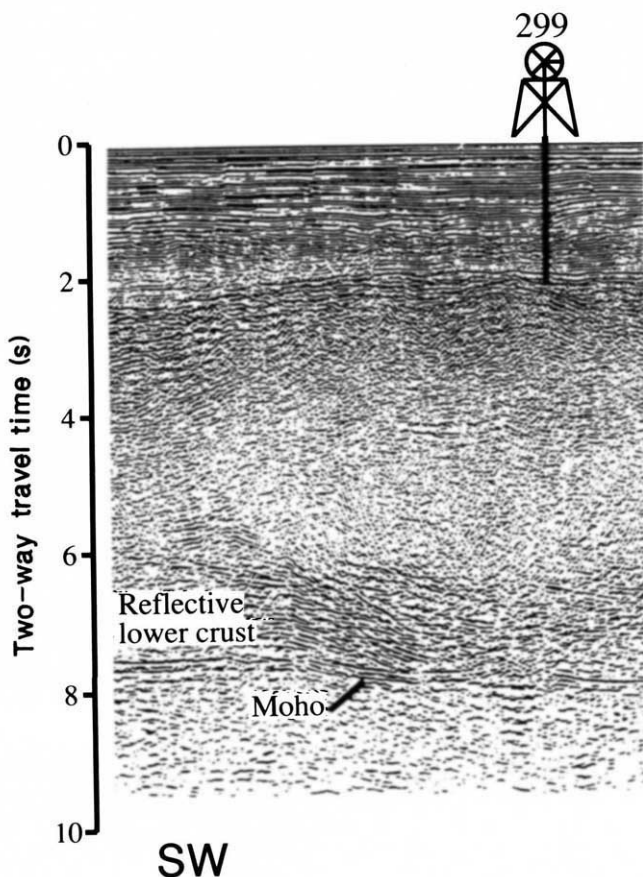
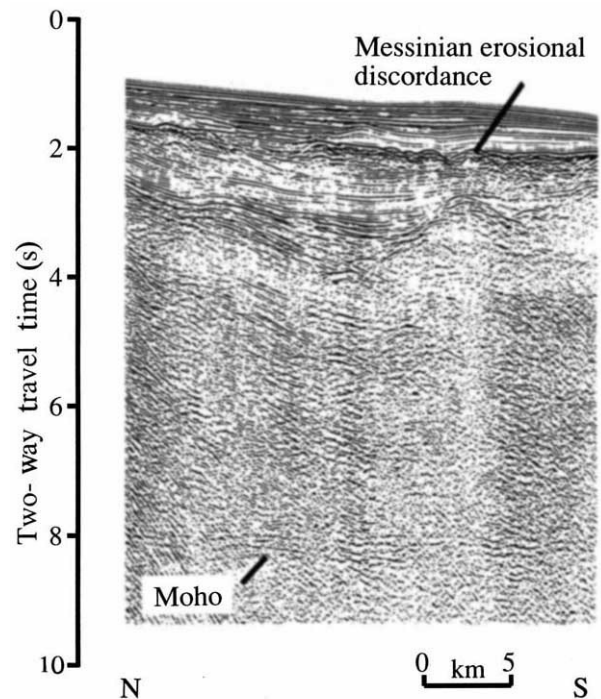


FIG. 2 Migrated 96-fold CDP line 819. The location of wells 299 and 419, the intersection of profile AB and the mid-point of ESP 6 are shown above the profile. The thick line at the end of the profile shows the velocity-TWT function deduced from modelling the data obtained along ESP 6 in the τ - p and X - T domains⁴⁰. The lower continental crust is associated with $V_p = 6.4$ –7.0 km s⁻¹ and is ~6 km thick.

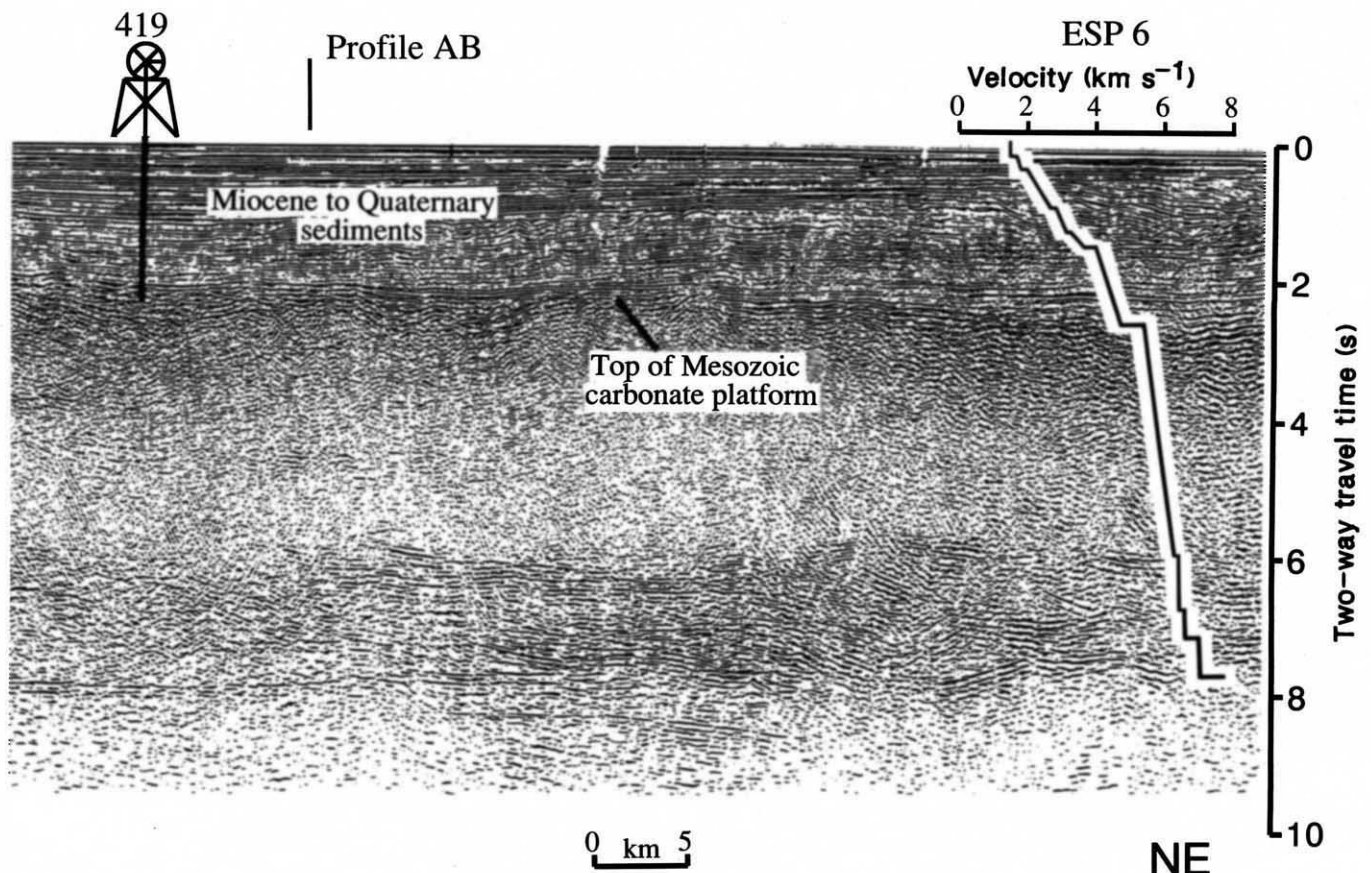
East Africa³³. Gravity modelling using Woollard's³⁴ velocity-density relationships is consistent with a crustal thinning of 11–14 km between profile I and ESP 6 and a further thinning of ~14 km towards the trough axis. Both seismic refraction and gravity anomaly data require a high-velocity dense lower crust (heavy shading), which we believe corresponds to the highly reflective lower crustal layer.

The crustal thickness (20 km) deduced from backstripping data from well 419 (the nearest well to profile AB) is in general agreement with that deduced from the seismic and gravity data (18–19 km), suggesting that much, if not all, of the 6-km-thick lower crustal reflective layer was already in the crust before extension. If it was added later, say by underplating²⁵, then the thickness of the stretched crust would be reduced to 12–13 km which corresponds to $\beta = 2.4$ –2.6. Thermal modelling shows that this value of β would yield a TTS of 2.5–2.7 km, which is not observed (Fig. 4). Another argument against underplating concerns the velocity of the lower crustal layer. As ESP 6 shows (Fig. 2), V_p is in the range 6.4–7.0 km s⁻¹, which is significantly smaller than the velocities of 7.2–7.4 km s⁻¹ reported from rifted margins such as the Baltimore Canyon trough³⁵ and Hatton Bank³⁶ which are believed to have been underplated.

The model in Fig. 5 implies that the highly reflective lower crustal layer is 13–15 km thick beneath the western end of profile AB and is absent beneath the Valencia trough. This thickness is in agreement with seismic refraction results along profile I²², although there is no evidence along this profile of the reflective nature of the lower crust. The nearest seismic reflection profile is the ECORS Pyrenees South line³⁷ (Fig. 1) which shows a 2-s TWT, or 6.5-km-thick layer (assuming a velocity of 6.6 km s⁻¹). The lower crust on this line has, however, been involved in the Pyrenean deformation. The western end of profile AB is located on Hercynian basement between the deformed zones of the Pyrenees and Betics. ECORS profiling of Hercynian crust in the Ardennes massif¹⁶ shows that the reflective layer is typically 4-s TWT, or 13 km thick, in agreement with the thickness



deduced at the western end of profile AB. If the 'normal' thickness of the lower crustal layer is 13 km, its absence beneath the trough suggests it has been either thinned by a greater amount than the upper crust or pervasively intruded by magmatic material so that the V_p of 7.8 km s⁻¹ (Fig. 2) corresponds to some form of 'crust-mantle mix'.



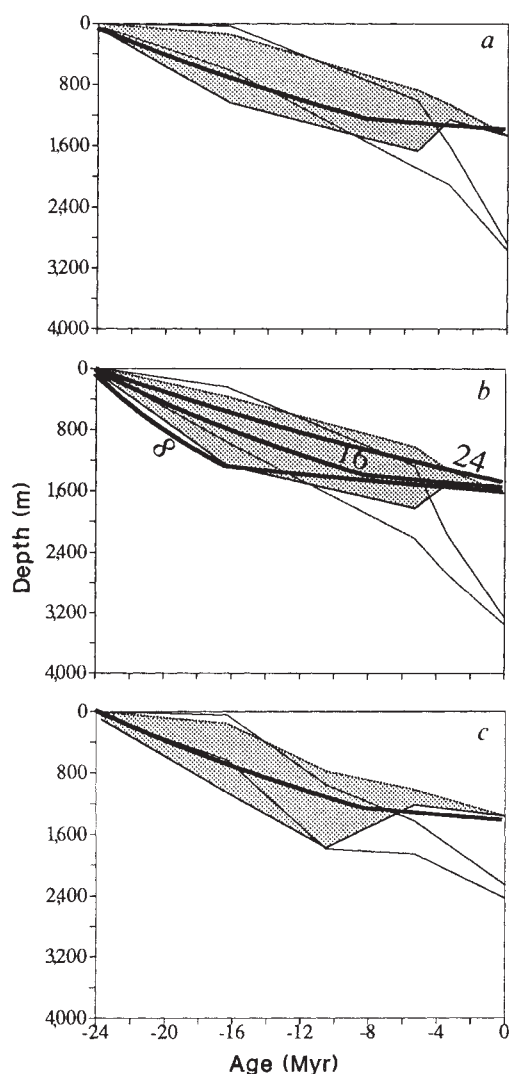


FIG. 4 Subsidence history of the flanks of the Valencia Trough based on backstripping stratigraphic data¹⁵ from the Castellon Wells a, 299, b, 419 and c, 466. Tectonic subsidence (shaded area), sediment accumulation (thin solid line) and calculated subsidence curves (thick solid line) based on finite-rifting models^{29,30} with rifting duration of 16 Myr and $\beta=1.40$ (well 299), rifting duration of 16 Myr and $\beta=1.45$ (well 466), and $\beta=1.55$ and rifting durations of 8, 16 and 24 Myr (well 419).

Our data do not support the view that the highly reflective lower crustal layer beneath the flanks of the Valencia trough is the result of the mid-Tertiary extensional event that formed the trough, even though this event seems to have been a protracted one. Elsewhere in Europe, the reflective layer apparently 'ponds' Hercynian structures and it has been suggested¹⁶ that they may post-date the Carboniferous. Moreover, the reflective layer is thinned on the ECORS Pyrenees South line³⁷ indicating a pre-Cretaceous age. Bois *et al.*¹⁶ suggested that the reflective layer was formed by re-equilibration of the crust following the Hercynian orogeny but, other data⁸ support the view that they originated by a mechanical transformation of the crust during extension. Our data suggest that a highly reflective lower crustal layer was already in the Iberian crust before rifting. The Valencia Trough, however, has few tilted fault blocks²⁴, a relatively low level of magmatic activity³⁸, and did not generate oceanic crust, so we cannot rule out the possibility that a more widespread extensional event, such as that associated with the Tethys, did not cause the highly reflective lower crust of Iberia and, perhaps, the rest of Europe. □

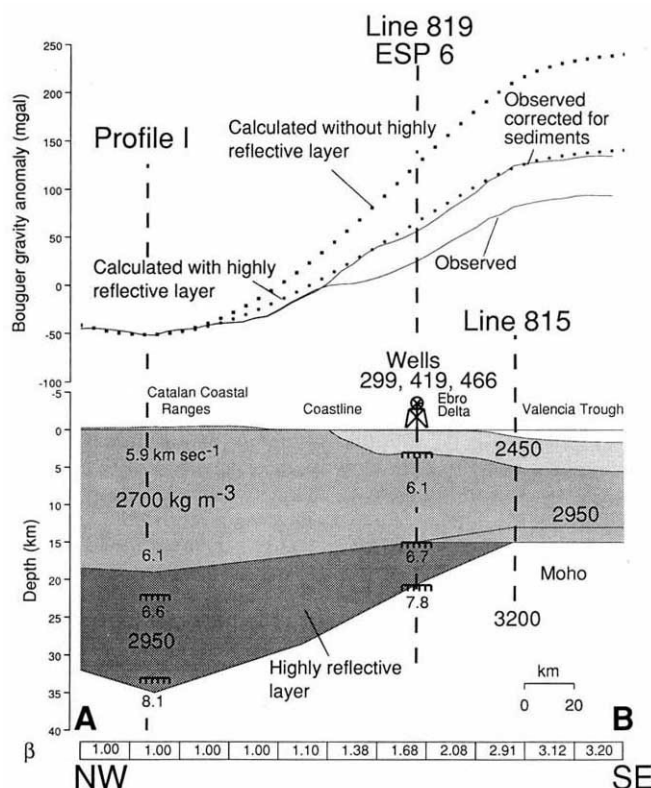


FIG. 5 Comparison of the observed Bouguer gravity anomaly (A.B.W. and M.T., manuscript in preparation) on profile AB to calculated profiles based on the seismically constrained crustal structure at profile I and ESP 6. The heavy shading shows the dense highly reflective lower crustal layer which thins toward the axis of the trough and thickens beneath Iberia. The medium shading shows the acoustically transparent upper crust. The light shading shows the distribution of sediments. The stretching factor β is based on the seismically and gravimetrically constrained crustal model and an initial (unstretched) crustal thickness of 31.2 km.

Received 15 June; accepted 8 October 1990.

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ACKNOWLEDGEMENTS. This work was supported by the NSF.

Use of learned odours by a parasitic wasp in accordance with host and food needs

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ADULT parasitic wasps need nectar or some other fragrant food source^{1,2}, but the effect of food odours on their behaviour has not been investigated to any extent³. Females of the parasitic wasp *Microplitis croceipes* learn volatile odours associated with host sites and use them to find hosts more effectively^{4,5}. We have now performed flight tunnel experiments that show that this wasp also uses airborne odours to find food sources. Females learn associatively and subsequently fly to volatile odours presented for smell while they are feeding on sugar water. Furthermore, they can learn two novel odours associated with separate host and food resources and then make an accurate choice between these odours on the basis of their relative host and food needs. This ability of parasitic wasps to link different odours with specific resources and then effectively to use them as cues for choice between competing needs is of fundamental and applied importance.

Females of *M. croceipes* fed on wildflower honey subsequently flew up-wind to the odour of honey, whereas naive females and females fed a pure aqueous sucrose solution both responded much more weakly (Fig. 1). To determine whether this oriented response to food was learned, female wasps were provided with

FIG. 1 Flight response of *M. croceipes* females to honey after no experience (naive), or feeding on sugar water or honey. Bars capped by different letters are significantly different, $P < 0.01$, Waller-Duncan K -ratio t -test; minimum significant difference, 21.9%, $n = 90$ wasps (5 replications, 6 wasps per replication per preflight treatment).

METHODS. *M. croceipes* were reared on larvae of *H. zea* fed an artificial diet. Females were allowed to mate and then held with a water supply in $30 \times 30 \times 20$ cm cages for 2 days after emergence. The 2-day-old females were each exposed to a drop of 20% sucrose water (10 μ l) or honey (Powers, wildflower) (50 mg) placed on the bottom of a polystyrene Petri dish (8×1.5 cm). Females were allowed to walk directly from a vial onto the test material and then to feed on the material for 5 seconds. This experience was repeated 3 times with 2-min intervals. The wind tunnel has been described⁴. All flight responses were tested at 26–28 °C at a wind speed of 56 cm s⁻¹ and at a light intensity of 2,000 lux. As an attractant source 50 mg of honey was pipetted onto a piece of Whatman number 1 filter paper suspended at the centre of the up-wind end of the tunnel. Females, tested individually, were released from a 4-dram shell vial 80 cm downwind and in the plume from the odour source. The behaviour of the females in the wind tunnel was observed until a complete flight occurred or 5 min had elapsed. A complete response involved the host-seeking flight behaviour described by Drost *et al.*⁴ and included approach and landing on the odour source. For each observation, a female was given two chances to make a successful flight. Flight tests were conducted 20 min post-experience.

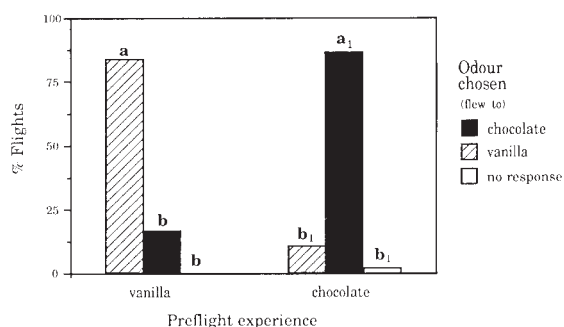


FIG. 2 Flight responses by *M. croceipes* females to vanilla or chocolate extract after smelling one of these odours while feeding on sugar water (preflight experience). Bars within the same treatment group capped by different letters are significantly different, $P < 0.01$, Waller-Duncan K -ratio t -test; minimum significant difference, 14.3%, $n = 64$ wasps (8 replications, 4 wasps per replication per preflight treatment).

METHODS. Females were reared and handled as described for Fig. 1. The two-day-old females were given a 10- μ l droplet of sugar water (20% sucrose) as described for Fig. 1. In addition, however, a 10- μ l droplet of either vanilla (Nielsen-Massey) or chocolate (McCormick) extract was placed 5 mm away from the sugar water for simultaneous smelling while feeding (care was taken that the females did not touch the extract). Wasps were permitted feeding/smelling training sessions of 5 s and sessions were repeated 5 times for each female with 2-min intervals between sessions. Twenty minutes after the final training session, females were released in the flight chamber as described in Fig. 1 and their response observed to a choice of vanilla extract (10 μ l) or chocolate (10 μ l) suspended 8 cm apart at the centre of the up-wind end of the chamber.

either one of two arbitrarily selected odours, vanilla or chocolate, while feeding on sucrose. A droplet of the vanilla extract or chocolate extract was placed near the sucrose solution so that the wasp could smell the odour while simultaneously tasting the sucrose, but was not allowed to taste or otherwise contact the odour. Females so trained showed a clear preference for the respective odour of their training when subsequently provided with a choice of these two odours in the flight chamber (Fig. 2). Females given the smell of vanilla or chocolate by the same procedure, but separately from feeding, did not respond to either odour.

In earlier studies, females presented with odours in association with hosts and host frass likewise linked these odours to hosts and flew to the odours in search of host resources^{4,5}. We conducted tests to determine whether females could learn and

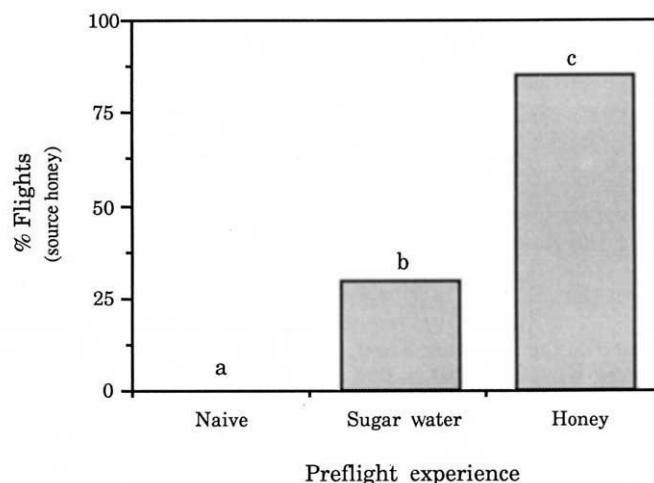
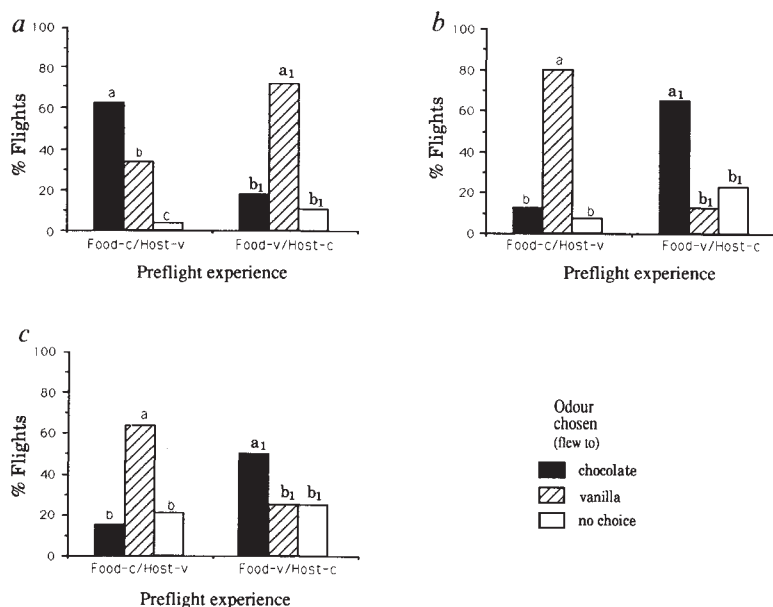


FIG. 3 Flight responses to vanilla or chocolate extract by hungry (a), well fed (b), or trained hungry then well fed (c) females, with preflight training indicated. Food-C/Host-V: females given training experience of chocolate-to-food and vanilla-to-hosts; Food-V/Host-C: females given training experience of vanilla-to-food and chocolate-to-hosts (the order of food-odour and host-odour training was alternated and the results combined). Bars within same treatment group capped by different letters are significantly different, $P < 0.01$, Waller-Duncan K -ratio t -test; minimum significant difference, 10.5% (a and b) and 16.3% (c); $n = 62$ wasps for each test group (6 replications, 6 wasps per replication per preflight treatment).

METHODS. Females were reared to 2 days old as described in Fig. 1. Hungry females were deprived of food (except water) until time for training and testing (2 days old). Well fed females were fed 20% sucrose water for 5 min when one day old. Training procedures were the same for hungry and well fed females. The training procedure to link odour (chocolate or vanilla) to food was as described in Fig. 2. The training procedure to link odour to hosts consisted of placing frass (1.4 mg from *H. zea* larvae fed on cowpea seedlings) and a third instar larvae on a filter paper plus 10 μ l of either chocolate or vanilla extract. Wasps were allowed to antennate the frass and sting the larva. This training was repeated 3 times for each female with 2-min intervals in between. The order of training for the food-odour and host-odour was alternated evenly. Forty minutes after the last training the wasps' flight responses to chocolate versus vanilla were tested in the flight chamber as described in Fig. 2. The



females used in Fig. 3c were each provided with a 3-min feeding session on 20% sucrose water 20 min after training and 20 min before testing.

differentially link two separate odours to their food and host foraging modalities and then if they could choose accurately between these learned cues on the basis of their current resource need. The females of one group (50% hungry and 50% well fed) were trained to associate chocolate with food and vanilla with hosts, whereas a second group of females (also 50% hungry and 50% well fed) were trained using these same two odours in reverse order. The training consisted of repeated presentation of the odour in association with either food or host (for details, see figure legends). The flight responses of hungry or well fed females trained in this way (Fig. 3a and b) were evaluated shortly (40 min) afterwards in a flight chamber by offering them a flight choice between vanilla and chocolate odours. The results in Fig. 3a and b indicate that the females were able to learn both odours successfully and could learn the combination in either direction (vanilla-to-hosts and chocolate-to-food, or chocolate-to-hosts and vanilla-to-food). Regardless of the direction of training, the hungry females showed a flight preference for the odour learned in connection with food (sucrose) and the well fed females preferred the odour learned as associated with hosts.

A final test was needed to ensure that the flight response was indeed a function of food and host needs, rather than a difference in how well the hungry or well fed wasps learned the odour associations. Therefore a group of hungry females were each trained to one of the host and food odour combinations (50% chocolate-to-food and vanilla-to-hosts; 50% vanilla-to-food and chocolate-to-hosts). Twenty minutes after training, each female was brought to the well fed state by a 3-min feeding session on pure aqueous sucrose solution, then their odour preferences were tested 20 min after the feeding (Fig. 3c). This group of well fed wasps, although trained while hungry, showed a preference for the host-associated odour like the well fed females (trained and tested while well fed) shown in Fig. 3b, in contrast to the hungry females (trained and tested while hungry) shown in Fig. 3a. These results confirm that the wasps readily learn both odour associations and can retrieve this information and respond in accordance with current need. Note that the alterna-

tive of training well fed wasps and testing them while hungry was not attempted. Memory decay during the extended period (~2 days) necessary between training and reaching a hungry state for testing makes this option impractical.

Learned responses to odours associated with hosts has been demonstrated for several types of parasitic wasp⁶⁻⁸, but our results are the first to show that parasitic wasps associatively learn and respond to odours affiliated with their food. Further, this finding reveals how parasitic wasps like *M. croceipes* can locate their food as well as polyphagous hosts under highly variable conditions. The female olfactory system constantly monitors the array of odours encountered during foraging. The associative occurrence of the odours with the different resources (hosts or food) are perceived and the linkages learned. Linked odours become cues for seeking those host or food resources and the rank order of responses to these learned cues can be varied in accordance with the relative levels of need for the different resources.

The finding of these remarkable capabilities by solitary parasitic wasps for processing olfactory information and adapting it for foraging advantages indicates a system that may rival that described for social honeybees⁹ and even rats¹⁰. This discovery increases our insight into invertebrate learning and ethology and should be of value in improving the use of parasitic wasps as biological control agents. □

Received 20 August; accepted 18 October 1990.

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Protein kinase A regulates chloride conductance in endocytic vesicles from proximal tubule

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REGULATION of ion transport by phosphorylation and G proteins occurs in several epithelial and non-epithelial cell plasma membranes¹⁻⁵. It is not known whether transporters on intracellular membranes are target sites for second messengers. Here we present direct evidence that a chloride conductance in endocytic vesicles from rabbit proximal tubule is activated by phosphorylation through a cyclic AMP-dependent protein kinase. To measure chloride transport, endocytic vesicles were labelled *in vivo* with a Cl^- -sensitive fluorescent indicator⁶⁻⁸. It was found that labelled endosomes contained an inward proton pump and a chloride conductance, but no ion-coupled chloride transport, and that the chloride conductance was regulated by protein kinase A. These results, taken together with measurements of chloride effects on ATP-dependent acidification, suggest that endosomal pH can be

controlled by phosphorylation of a stilbene-sensitive conductive chloride transporter.

The apical plasma membrane of proximal tubule in mammalian kidney undergoes rapid turnover by a cycle of membrane insertion and retrieval⁹. By use of an *in vivo* labelling technique^{10,11}, it was shown that endocytic vesicles labelled with FITC-dextran (relative molecular mass 10,000 (M_r 10K)) contained a functional water channel and an electrogenic, inward proton-pumping H^+ -ATPase¹². It has been proposed that acidification of endosomal, pre-lysosomal and lysosomal vesicles might require a Cl^- conductance to shunt the interior positive diffusion potential produced by active proton pumping^{13,14}. Regulation of such a Cl^- channel would provide a direct means to control internal pH and thus important cellular degradative and transport functions. Other mechanisms that could in principle regulate the kinetics of endosomal acidification are the number/activity of proton pumps¹⁵, ion pumps^{16,17} and passive proton conductance¹⁸.

To measure rapid Cl^- influx into vesicles arising from endocytosis of apical membrane, 1–2-kg New Zealand white rabbits were intravenously infused over 1 min with SPQ (6-methoxy-N-3-sulphopropyl quinolinium), a membrane-impermeant, Cl^- -sensitive indicator whose fluorescence responds in <1 ms to a change in Cl^- concentration⁶. The rabbit was killed after 15 min. The superficial renal cortex was dissected, homogenized and centrifuged to give a crude microsomal pellet containing the SPQ-labelled endosomes. As described below, double-labelling studies showed that SPQ co-localized in

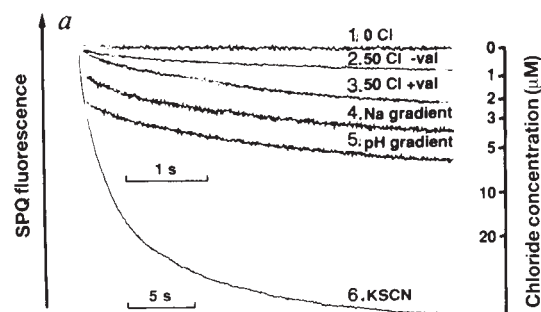
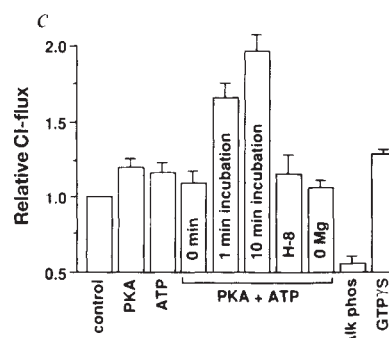
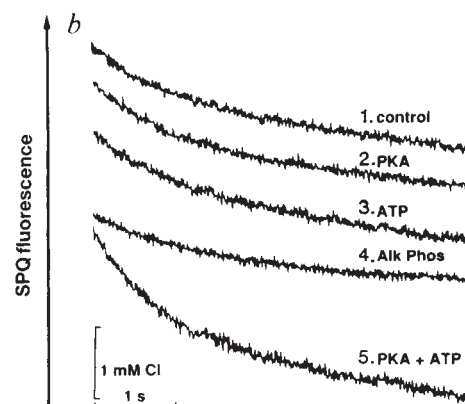


FIG. 1 Time-course of Cl^- influx in endocytic vesicles from rabbit proximal tubule. **a**, Microsomes in buffer I (see Methods) were mixed with an equal volume of: curve 1, buffer I (Cl^- absent); curve 2, 100 mM mannitol, 100 mM KCl, 5 mM potassium phosphate, 1 mM magnesium acetate, pH 7.4 (buffer III) (valinomycin absent) to give a 50 mM Cl^- gradient; curve 3, buffer III with valinomycin present; curve 4, 50 mM potassium isethionate, 50 mM KCl, 50 mM NaCl, 5 mM potassium phosphate, 1 mM magnesium acetate (valinomycin present) to give inward gradients of Na^+ and Cl^- . Curve 5, Microsomes in buffer I in which 25 mM NaHCO_3 replaced K isethionate (bubbled with 5% CO_2) were mixed with buffer III containing 5 mM NaHCO_3 at pH 6.5; curve 6, 250 mM KSCN. Curves 4 and 5 were displaced for visual clarity. **b**, Microsomes (1 mg ml^{-1}) were incubated for 10 min at 23 °C with ATP (0.5 mM) and/or PKA (10 U ml^{-1}) before stopped-flow experiments. Where indicated, microsomes were pretreated with alkaline phosphatase (100 U ml^{-1} , 10 min) and washed once. Experiments were as in **a**, curve 3, in which the Cl^- gradient was 50 mM. Curves are the average of ten experiments performed on one microsome preparation. **c**, Mean $\pm$ s.e.m. for paired studies (6–10 separate preparations, 10 experiments averaged for each preparation) as in **b**. H-8 ($100 \mu\text{M}$) was added 5 min before PKA and ATP addition. Unless otherwise indicated, incubation time was 10 min. 0 Mg indicates replacement of Mg^{2+} by 2 mM EDTA. GTP- γ -S concentration was 0.2 mM.

METHODS. Rabbits were infused with 200 mg SPQ and killed after 15 min. The renal arteries were perfused with buffer I (100 mM potassium isethionate, 100 mM mannitol, 1 mM magnesium acetate, 5 mM potassium phosphate, pH 7.40) or II (300 mM mannitol, 5 mM HEPES/Tris buffer, 1 mM magnesium acetate, pH 7.40). Superficial renal cortex was dissected in 1-mm slices and homogenized by 10 strokes of a Potter-Elvehjem homogenizer in buffer I or II and centrifuged (1,000g for 10 min) to remove large debris. The supernatant was centrifuged (100,000g for 30 min) and washed once to give a microsomal pellet. The pellet was homogenized using



a 25-gauge needle and used within 2 h. A Hi-Tech SF-51 apparatus was used for stopped-flow studies in which equal volumes of the microsomal suspension (0.1–0.2 mg membrane protein per ml) were mixed with solutions containing Cl^- . The excitation source was a stabilized 100-watt Hg-Xe arc lamp in series with a six-cavity $365 \pm 5 \text{ nm}$ bandpass filter. Fluorescence was detected through a 420-nm cut-on filter. There was no scattered light or inner filter effect. Sample acquisition rate was 2 ms per point. The initial rate of Cl^- influx (J_{Cl}) was calculated from the initial slope of the fluorescence decrease, the calibration between SPQ fluorescence and Cl^- concentration, and the fluorescence at zero Cl^- and infinite Cl^- (after KSCN addition) as described previously⁶. Unless stated otherwise, microsomes contained valinomycin, 5 μg per mg membrane protein.

vesicles with FITC-dextran, a probe shown previously to label endosomes arising from proximal tubule apical membrane with >95% selectivity¹⁹. Chloride transport was measured from the time-course of SPQ fluorescence, when microsomes not containing Cl^- were subjected to inwardly directed Cl^- gradients in a stopped-flow apparatus. The time resolution was ~ 1 ms. An important advantage of the *in vivo* labelling technique was that information about Cl^- transport could be obtained selectively in labelled endocytic vesicles present in a crude microsomal suspension that was prepared rapidly and subjected to minimal membrane manipulation.

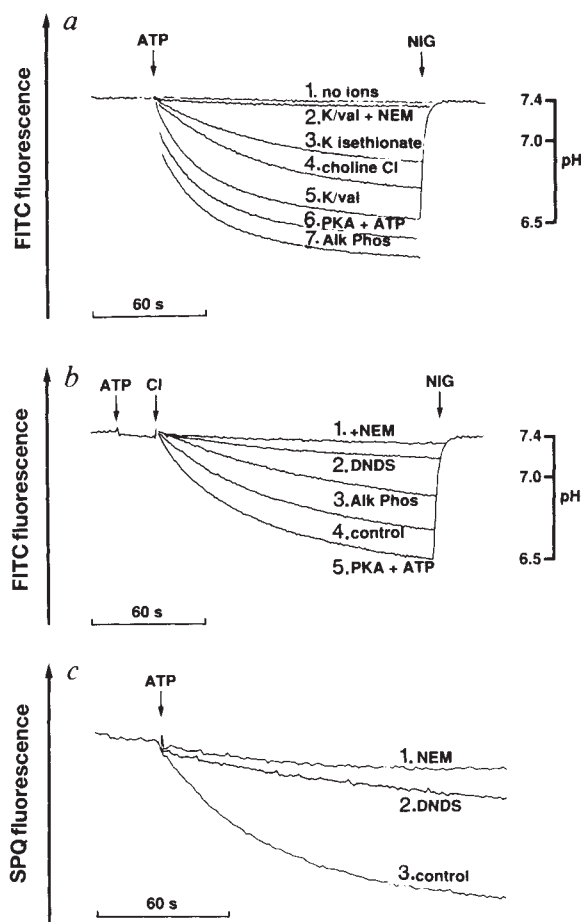


FIG. 2 Time course of pH and Cl^- concentration in endocytic vesicles in response to ATP addition. **a**, Microsomes containing endocytic vesicles labelled with FITC-dextran (10K) were prepared by intravenous infusion of 500 mg FITC-dextran and homogenization as described in the legend to Fig. 1. ATP (1 mM) and nigericin (5 μM) were added where indicated. FITC fluorescence (excitation 485 nm, emission >520 nm) was monitored continuously in a stirred cuvette in an SLM 48000 MHF fluorimeter. Absolute pH was determined from pH calibration curves as described previously¹². Curve 1, solutions (inside and outside) contained buffer II (no permeant ions). Curves 2 and 5, solutions contained buffer I (valinomycin present) in the absence (5) or presence (2) of 0.5 mM *N*-ethylmaleimide (NEM). Curve 3, solutions contained buffer I with valinomycin absent. Curve 4, solutions contained 100 mM choline chloride, 100 mM mannitol, 5 mM HEPES/Tris buffer, 1 mM magnesium acetate, pH 7.4 (buffer IV). Curves 6 and 7 same as 5 except for prior 10 min treatment with PKA (10 U ml^{-1}) + ATP (0.5 mM) or alkaline phosphatase (100 U ml^{-1}). **b**, ATP (1 mM), choline chloride (40 mM) and nigericin (5 μM) were added where indicated. Curve 1, 0.5 mM NEM present. Curve 2, 0.1 mM DNDS present. Curve 3, microsomes pretreated with 100 U ml^{-1} alkaline phosphatase. Curve 4, control. Curve 5, microsomes preincubated with 10 U ml^{-1} PKA + 0.5 mM ATP. **c**, SPQ fluorescence was monitored in microsomes containing SPQ in buffer II (no ions) suspended in buffer IV. Where indicated, 1 mM ATP was added. Curve 1, 0.5 mM NEM present. Curve 2, 0.1 mM DNDS present. Curve 3, control.

Figure 1a shows the time course of SPQ fluorescence in endocytic vesicles subjected to Cl^- gradients. Each experiment was internally calibrated by addition of 250 mM potassium thiocyanate which quenches >99% of SPQ fluorescence⁸ (Fig. 1a, curve 6, see legend). Fluorescence did not change in the absence of external Cl^- (Fig. 1a, curve 1). Fluorescence decreased in response to a 50 mM Cl^- gradient due to Cl^- influx and quenching of entrapped SPQ. The initial rate of Cl^- influx increased from 0.11 ± 0.03 ($\pm$ s.d., $n = 5$) to 0.53 ± 0.04 ($n = 18$) mM s^{-1} by addition of the potassium-selective ionophore valinomycin, indicating conductive Cl^- entry (Fig. 1a, curves 2 and 3). Chloride influx was inhibited 50% by the stilbene inhibitor 4,4'-dinitrostilbene-2,2'-disulphonic acid (DNDS, 100 μM , not shown). Chloride influx was not altered by Na^+ or HCO_3^-/pH gradients (Fig. 1a, curves 4 and 5), indicating absence of ion-coupled Cl^- transport mechanisms.

Figure 1b shows the influence of ligands added to the microsomal suspension containing labelled endosomes that have an outward-facing regulatory site. Potassium and valinomycin were present to eliminate diffusion potentials so that Cl^- transport was rate-limiting. Initial Cl^- influx was increased by incubation with the purified catalytic subunit of protein kinase A (PKA) and ATP; there was little effect of ATP or PKA added alone. Treatment with alkaline phosphatase decreased Cl^- influx. Results from a series of paired studies are given in Fig. 1c. The increase in Cl^- influx was >50% complete after 1 min of incubation with PKA and ATP. Chloride influx was not increased when membranes were incubated with the PKA inhibitor H-8 (ref. 20), when the incubation time with ATP and PKA was 0 min, and when Mg^{2+} was replaced by EDTA. There was little effect of maximal GTP- γ -S. These results provide evidence that conductive Cl^- influx is regulated by transporter phosphorylation catalysed by PKA.

The effects of Cl^- on ATP-dependent acidification were examined in endosomes labelled with FITC-dextran. Acidification of endosomes in response to external ATP was blocked by removal of permeable ions or by NEM (*N*-ethylmaleimide) (Fig. 2a, curves 1 and 2). In the absence of Cl^- submaximal acidification in the presence of K^+ (Fig. 2a curve 3) was increased by valinomycin (Fig. 2a, curve 5). Chloride in the absence of permeable cations gives strong acidification, although less than that of K^+ /valinomycin. Acidification was not influenced by ouabain in the presence of Na^+ and K^+ (data not shown). Acidification in the presence of K^+ and valinomycin was not influenced by prior phosphorylation with PKA and ATP (Fig. 2a, curve 6), or by prior dephosphorylation with alkaline phosphatase²¹ (Fig. 2a, curve 7), indicating that membrane phosphorylation did not alter proton pump function directly.

Figure 2b shows ATP-dependent acidification under conditions in which Cl^- was the major permeable ion, so that differences in acidification could be attributed to Cl^- conductance. Phosphorylation of endosomes by PKA and ATP enhanced ATP-dependent acidification. Treatment with alkaline phosphatase caused a remarkable decrease in acidification. Acidification was strongly inhibited by inhibition of the proton pump (NEM) or Cl^- conductance (DNDS). We conclude that Cl^- is an effective counterion for control of ATP-dependent acidification and that PKA acts on a stilbene-sensitive Cl^- conductance.

We confirmed that SPQ-labelled apical endosomes and that PKA effectively phosphorylated endosomal proteins under experimental conditions. Figure 2c demonstrates functional colocalization of the H^+ -ATPase and Cl^- conductance; ATP addition drove inward Cl^- movement because of depolarization of the interior membrane potential by the proton pump. Inward Cl^- movement was blocked by NEM or DNDS. Morphological co-localization of FITC-dextran and SPQ was shown by visualizing endosomes on poly-L-lysine coated coverslips by epifluorescence microscopy. More than 96% of endosomes labelled with SPQ (excitation 365 ± 5 nm, emission 430 ± 20 nm) or with FITC-

dextran (excitation 480 ± 5 nm, emission >520 nm) were labelled with both; there was no spectral overlap of the fluorescence signals. Similar results were obtained by examination of unfixed, freshly cut thick cortical slices by epifluorescence confocal microscopy.

Membrane protein phosphorylation was measured from incorporation of [32 P]ATP into a purified suspension of endocytic vesicles²² by autoradiography on SDS-PAGE²³. Under the conditions given in Fig. 1, incubation with PKA for 10 min increased phosphorylation of proteins at 32, 55, 68 and 74K by >10 -fold; phosphorylation was $>75\%$ complete in 1 min and was $>90\%$ blocked by H-8 (*N*-2(methylamino)ethyl-S-

isoquinoline-sulphonamide) or by replacement of Mg^{2+} by EDTA.

Taken together, the results provide evidence that a stilbene-sensitive Cl^- conductance in endocytic vesicles is regulated by cAMP-dependent protein kinase. Phosphorylation of a Cl^- conductance would provide a simple means to control pH as endosomes mature by fusion events or movement along a continuous reticulum²⁴. Abnormal regulation of an intracellular Cl^- conductance could be a component of the disease cystic fibrosis, in which many cellular processes are affected. Our findings also demonstrate that an ion transporter on an intracellular membrane may be a target site for a second messenger. □

Received 28 August; accepted 4 October 1990.

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ACKNOWLEDGEMENTS. We thank W. Reenstra and I. Sabolic for help in endosome purification and [32 P]ATP studies. A.S.V. is an established investigator of the American Heart Association. This work was supported by the National Institutes of Health, National Cystic Fibrosis Foundation and American Heart Association.

Transfer of diabetes in mice prevented by blockade of adhesion-promoting receptor on macrophages

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INSULIN-dependent diabetes mellitus (IDDM) is a disease with an autoimmune aetiology. The non-obese diabetic mouse is a good spontaneous animal model of the human disease, with IDDM developing in 50-80% of female mice by the age of 6 months^{1,2}. The disease can be transferred by splenic T cells from diabetic donors and is prevented by T-cell depletion^{3,4}. The mechanism(s) by which the β cell is specifically destroyed is not known, but T cells and macrophages have both been implicated, based on the presence of macrophages in the infiltrated islet and the ability of chronic silica treatment to prevent disease^{5,6}. The monoclonal antibody 5C6 is specific for the myelomonocytic adhesion-promoting type-3 complement receptor (CR3 or CD11b/CD18)⁷ and does not bind to T cells. Here we show that blockade of macrophage CR3 *in vivo* prevents intra-islet infiltration by both macrophages and T cells and inhibits development of IDDM. We conclude that both T cells and macrophages have an essential role in the onset of IDDM.

We have previously shown that the monoclonal antibody 5C6 can prevent the migration of macrophages to inflammatory sites⁷. This is accomplished by inhibiting transendothelial migration without depleting cells from the marrow or vascular compartments and without inhibiting the secretory capacity of the macrophage. In addition we showed that sensitization of T cells

and, by inference, antigen presentation by macrophages was also unaffected by this antibody⁸.

To determine the role of macrophages in β -cell destruction, the ability of spleen cells from diabetic donors to transfer disease into 5C6-treated recipients was investigated.

Although male non-obese diabetic (NOD) mice develop insulinitis, overt diabetes is less than 10% in most colonies. This makes them suitable recipients in which to induce diabetes by diabetic spleen-cell transfer and therefore useful for establishing the part played by different cell populations in the development of disease.

Sub-lethally irradiated 2-3-month-old male NOD recipient mice were injected with 5C6 one day before cell transfer; antibody injections were repeated the next day and three times per week throughout the experiment. Therefore all macrophages of both donor and host origin were exposed to circulating 5C6. In one group, however, treatment was stopped after 10 days and in another, it did not start until day 10 (see legend to Fig. 1). Sub-lethally irradiated control animals were given either phosphate-buffered saline (PBS) or YTH 3.2.6, an isotype-matched control monoclonal antibody⁹. Glucose in blood and urine was monitored in all mice. Figure 1a shows the onset of hyperglycaemia following the transfer of 2×10^7 diabetic spleen cells into untreated, control-antibody-treated, or 5C6-treated normoglycaemic male NOD recipients. The incidence of overt diabetes in 5C6-treated animals was significantly reduced (from 93% to 8%; $P = 0.00018$, Fisher's exact test). Serum samples taken at intervals confirmed that a high level of circulating monoclonal antibody in recipients was correlated with protection.

From Fig. 1b it can be seen that delaying the administration of 5C6 until 10 days after transfer did not protect mice from subsequent hyperglycaemia. Furthermore, this figure shows that antibody blockade of the receptor has to be maintained to prevent disease in that suspension of 5C6 treatment after 10 days delayed but did not prevent the onset of IDDM. This confirms our observation that once the circulating level of 5C6 falls, mobilized macrophages are able to recruit T cells to the pancreas.

Four weeks after transfer, when all the untreated mice were already overtly diabetic, both these and the 5C6-treated animals

TABLE 1 Immunohistochemical analysis of pancreatic islets from 5C6-treated and untreated NOD mice

Treatment	No. mice examined	No. islets analysed	Islets with + peri-islet infiltration (%)	Islets with ++ intra-islet infiltration (%)	Residual islets (%)
Double staining for insulin and F4/80:					
5C6 + d.sp.	7	216	84*	1†	20
PBS + d.sp.	4	109	47	29	84
Non-d.sp. only	4	95	98	8	8
Double staining for insulin and CD3:					
5C6 + d.sp.	7	129	76‡	9§	19
PBS + d.sp.	4	43	38	52	72
Non-d.sp. only	4	89	87	2	23

Irradiated male NOD mice were given 2×10^7 spleen cells from diabetic donors (d.sp.) or from non-diabetic syngeneic age-matched males. Pancreatic cryostat sections were double-stained with guinea-pig anti-insulin antibody and either rabbit anti-F4/80 (ref. 10) which recognizes mature mouse macrophages (prepared by P. Dri) or KT3, a rat anti-mouse CD3 monoclonal antibody (prepared by K. Tomonari). Those islets that did not contain immunoreactive insulin are designated residual and calculated as a percentage of the total analysed.

* Minimal infiltration of 0–20 F4/80⁺ cells peri-islet.

† Maximal infiltration, namely 11 or more F4/80⁺ cells intra-islet.

‡ 0–10 CD3⁺ cells peri-islet.

§ More than 6 CD3⁺ cells intra-islet.

Grading of peri-islet and intra-islet infiltration was calculated as a percentage of those islets that remained. PBS, Phosphate-buffered saline.

were killed and the histology of the pancreata compared. It was found that administration of 5C6 throughout the experiment had preserved insulin-containing islets intact. Staining for class II or the macrophage-specific F4/80 antigen¹⁰ revealed only a predominantly peri-islet infiltrate, comparable to that seen in age-matched irradiated males reconstituted with spleen cells from non-diabetic NOD mice (see Fig. 2 and Table 1, showing

data for F4/80 staining). Untreated or control antibody-treated males, however, had very few intact islets remaining and all islets showed advanced intra-islet infiltration of class II⁺ cells (Fig. 2) and CD3⁺ T cells (Table 1).

Histological scoring of islet infiltration is shown in Table 1. Note that 84% of islets in control mice were scored as residual, with intact glucagon and somatostatin but no insulin

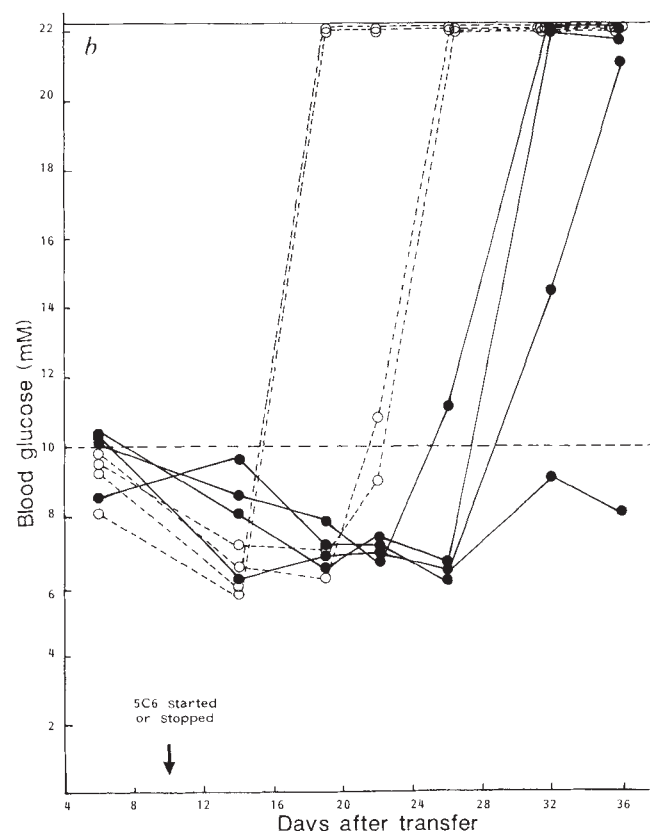
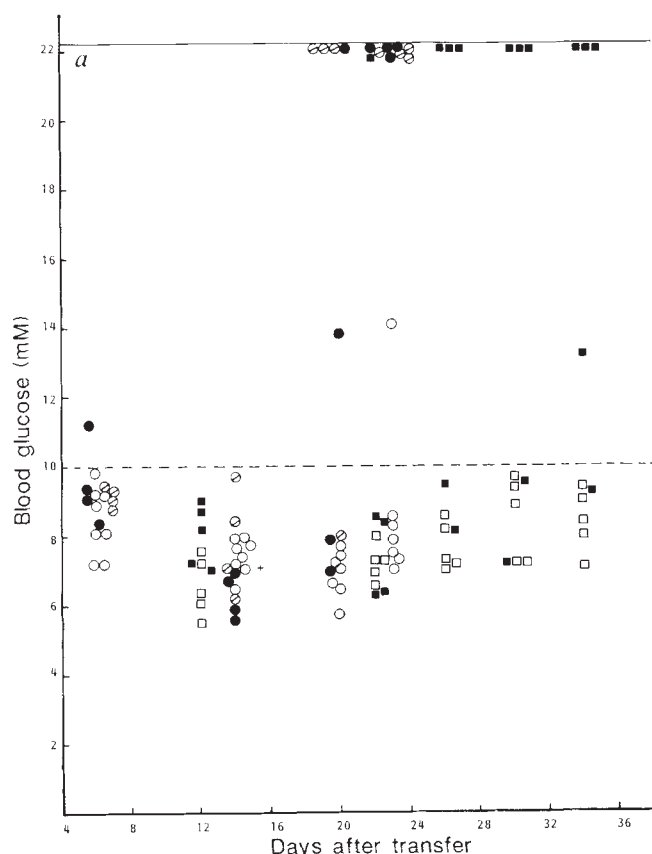


FIG. 1 The effect of 5C6 on the transfer of diabetes. *a*, Groups of 4–7 male NOD/CRC mice of 2–3 months old were injected on day –1 with 500 μ g 5C6 (open symbols), or control antibody YTH 3.2.6 (hatched symbols), or PBS (closed symbols). The initial injection was intravenous and all subsequent injections were intraperitoneal. On day 0, all mice were irradiated (650 rads; cobalt source) and given 2×10^7 spleen cells from diabetic donors intravenously. On day 1 and then three times every week, 5C6 or control antibody was administered. Data are from two separate experiments (circles

and squares) in one of which (circles) the mice were killed at 4 weeks for histological examination of the pancreata. The mouse designated by a cross died from unknown causes but was not diabetic. *b*, A further group of mice were given 5C6 for 10 days only (●). Another group was not started on this antibody treatment until day 10 (○). Blood glucose was assessed using a glucometer (Ames; limit of detection at the maximum reading of 22.2 mM) and urine was tested with Diastix (Ames). Mice were considered diabetic if blood glucose was consistently above 10 mM and the urine test was positive.

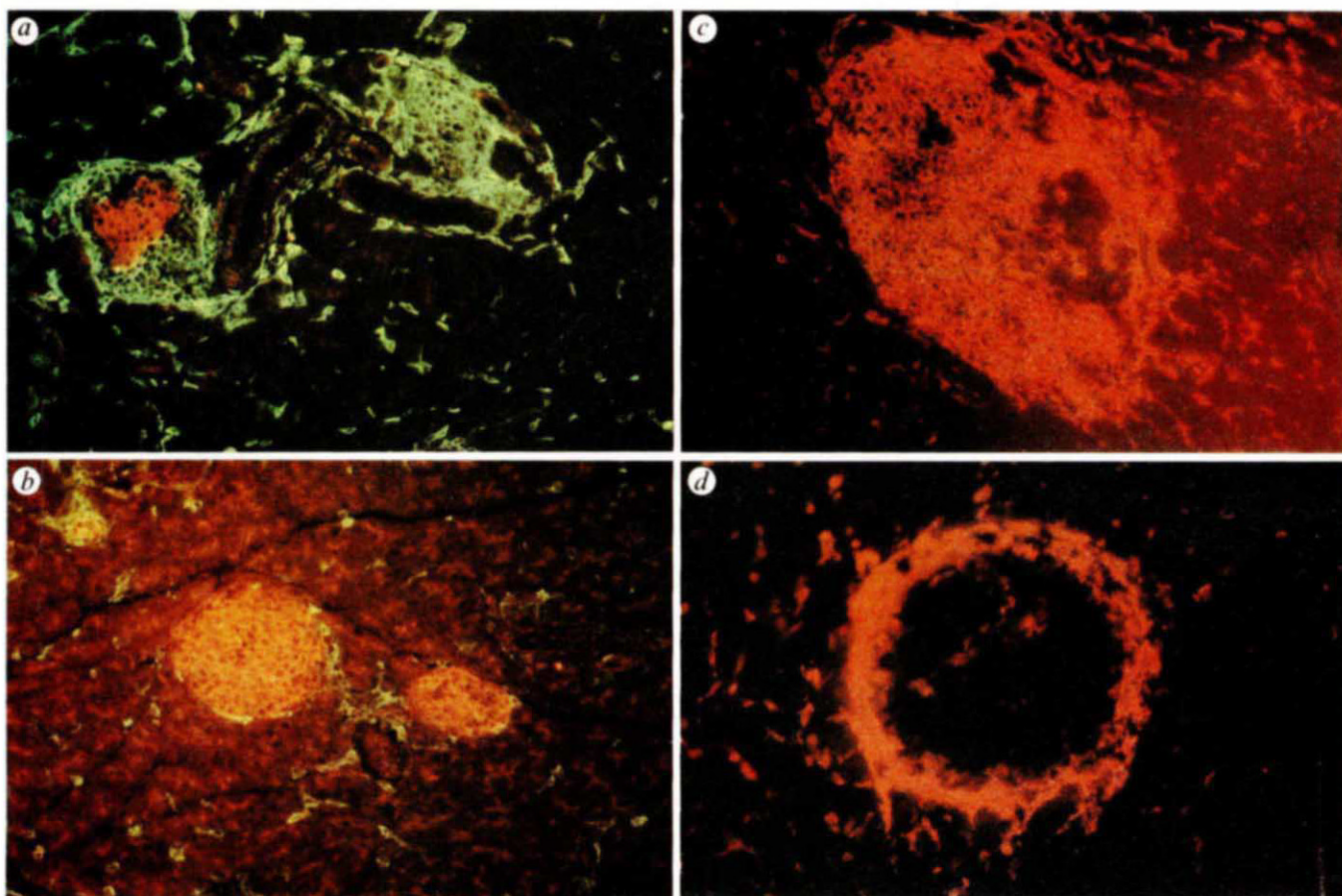


FIG. 2 Histology of islets showing protective effect of 5C6. Photomicrographs of pancreatic cryostat sections double-stained for insulin (red) and F4/80 (green) from control untreated mice (a) and 5C6-treated mice (b) four weeks after adoptive transfer of diabetic spleen cells. In c (control mice) and d (5C6-treated mice), sections were stained with polyclonal rabbit anti-F4/80 alone (red). Islets from the control group (a and c) showed

uniform intra-islet infiltration by F4/80⁺ cells, with loss of insulin and normal islet morphology. The 5C6-treated mice showed very little intra-islet infiltration (Table 1) but had a mixture of completely intact islets (as in b) or islets showing peri-islet infiltration (as in d). This pattern of infiltration is similar to that found in age-matched male NOD mice after the adoptive transfer of normal spleen cells. Magnification in a and b, 100 $\times$; in c and d, 200 $\times$.

immunoreactivity, whereas only 20% of islets in 5C6-treated mice showed comparable destruction with complete loss of insulin. Staining for CD3 showed that with no macrophage infiltrate, intra-islet penetration by T cells was also inhibited (Table 1).

From these data, we suggest that 5C6 exerts its effect by suppressing the early phase of macrophage migration into the islets, upon which later T cell-mediated events depend. Monocyte recruitment is followed by bulk migration of both sensitized and non-sensitized T cells to the islets. Analysis of the histology in the 5C6-treated and control mice supports such a scheme where inhibition of macrophage recruitment to the pancreas prevents later intra-islet T cell accumulation.

The mechanism by which the macrophage promotes T-cell recruitment is unclear but there are several possibilities. Local production of cytokines (for example, tumour necrosis factor α and interleukin 1) by macrophages may affect expression of adhesion molecule VCAM-1 on vascular endothelium, thereby facilitating mononuclear cell migration¹¹.

Tumour necrosis factor- α in the presence of γ -interferon increases class I expression on islet cells¹², which may itself play a part in β -cell destruction^{13,14}. Macrophage accumulation in the pancreas may well have a major role in antigen processing and presentation to T cells^{6,15} but in addition, they may themselves destroy β cells by release of cytokines or free radicals¹⁶. By preventing the CR3-dependent monocyte migration, 5C6 treatment also inhibits the passage of lymphocytes into the islets

and thereby arrests the subsequent cascade of cellular interactions which culminate in the destruction of insulin-containing β cells. Later administration of 5C6, at a time when recruitment is already advanced¹⁴, failed to affect the onset of hyperglycaemia. This is to be expected because 5C6, which lethally potentiates acute bacterial infections, fails to do so for listerial infections once granuloma formation has occurred¹⁷. But these observations do not preclude a direct role for macrophages or their secretory products in β -cell destruction once they have aggregated at the target site.

It is significant that 5C6 prevents some infiltration by T cells as well as macrophages, for CR3 is not expressed on T cells. The effect of 5C6 on T-cell recruitment is therefore probably indirect, with the macrophage being implicated as there is little evidence of other CR3-bearing cells (neutrophils and natural killer cells) in infiltrates of diabetic pancreata. After administration of 5C6 is stopped, blockade of CR3 is reversed, macrophage migration to the islets becomes possible, and T-cell recruitment and β -cell destruction ensues. This indicates that CD4⁺ and/or CD8⁺ effectors in the transfer inoculum are still potentially agents for destruction once macrophage migration to the islet becomes possible.

These data highlight the essential contribution of both macrophages and T cells to the onset of a complex autoimmune disease and suggest that therapeutic strategies should consider both cell types and their diverse mechanisms of tissue destruction. □

Received 6 August; accepted 19 October 1990.

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ACKNOWLEDGEMENTS. This work was supported by the Medical Research Council, the Arthritis and Rheumatism Council, the British Diabetic Association and the Wellcome Trust. We thank Professor Roitt and P. Crocker for discussion.

Strong xenogeneic HLA response in transgenic mice after introducing an $\alpha 3$ domain into HLA B27

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THE pronounced response by mouse T cells to the major histocompatibility complex (MHC) class I antigens of the same species is characterized by a relatively large fraction of responding cells (reviewed in ref. 1). Responses to MHC class I alleles of other species are, however, generally much weaker²⁻⁵. T lymphocytes are positively selected on thymic MHC antigens, resulting in a T-cell repertoire with strong alloreactivity⁶. This has been explained in terms of a mouse T-cell repertoire that is not efficiently selected for recognition of HLA molecules owing to the absence of HLA in mice. Here we show that mice transgenic for HLA mount a T-cell response against allogeneic HLA that is no better than in normal mice. We decided instead to test whether the mouse accessory molecule Lyt-2 on cytotoxic T lymphocytes could interact efficiently with the $\alpha 3$ domain of HLA. To do this, we replaced the $\alpha 3$ domain of HLA-B27 by a murine $\alpha 3$ domain in a gene construct used to produce transgenic mice, and then used the spleen cells from these mice to stimulate normal mouse T cells. Under these conditions cytotoxic T lymphocytes were generated with the same frequency against xenogeneic HLA-B27 determinants as against allogeneic mouse class I antigens. These findings indicate that the normally weak xeno-MHC response is due to the inefficient interaction of the murine Lyt-2 accessory molecule with HLA class I, and not to limitations of the mouse T-cell repertoire.

To study the ability of mouse T lymphocytes to generate a primary cytotoxic T-cell response against HLA class I antigens, spleen cells from C57Bl/6 or (C57Bl/6 $\times$ DBA/2) F_1 mice were stimulated *in vitro* against splenocytes from HLA-transgenic mice. HLA-transgenic spleen cells were used instead of HLA gene-transfected cell lines for stimulation because spleen cells are superior stimulators for the generation of cytotoxic T lymphocytes (CTL). These CTL were tested on Bc20 fibroblasts transfected with HLA class I genes. Only a weak or no CTL response was generated against the HLA molecules, whereas aliquots from the same responder populations always produced a strong primary CTL response against allogeneic H-2 class I antigens (Table 1). A weak but significant primary response against HLA-transgenic spleen with a CTL precursor frequency of $1/10^5$ has been reported⁷. We then used HLA-transgenic mice as responders to investigate whether such mice would have a higher frequency of allo-HLA reactive cells. Again, we observed no significant primary CTL response. Several transgenic responder/stimulator combinations were used, including B7 anti-B2705, B2705 anti-B7, Cw3 anti-B2705, Cw3 anti-B2702, B2705 anti-Cw3, and B13 anti-B2705. Some examples are shown in Table 1.

These results are consistent with previous studies in which HLA-Cw3 transgenic mice responded no better than normal mice to HLA-A2-transfected fibroblasts⁸. The data suggest that the presence of HLA in the transgenic mice does not select the mouse T-cell repertoire for better recognition of allo-HLA molecules. The Lyt-2 (mouse) or CD8 (human) antigen on the surface of CTL has been proposed to interact with the target MHC class I molecule, so increasing the avidity of the CTL for the target cells⁹⁻¹¹. In addition, several reports have suggested that the Lyt-2 molecule interacts with the $\alpha 3$ domain of MHC class I molecules¹²⁻¹⁶.

We therefore investigated the possibility that the low xeno-MHC response could be due to a species-barrier in the interaction of the mouse Lyt-2 accessory molecule with the HLA class

TABLE 1 Lack of primary anti-HLA CTL response in HLA-transgenic mice

Responder	Stimulator	Target		Stimulator	Target	
		Bc20.Cw3	bc20.B27/ $\beta 2$ (% lysis)		1T22-6	1T.K ^k (% lysis)
B6D2F ₁	tg B2705	6.8	12.5	C3H	7.0	46.3
tg Cw3	tg B2705	8.9	10.8	C3H	3.1	34.9
tg B7	tg B2705	5.3	12.6	C3H	9.5	53.6
tg B13	tg B2705	5.8	9.1	C3H	6.8	57.6
B6D2F ₁	tg Cw3	12.2	9.2	C3H	4.5	40.5
tg B2705	tg Cw3	12.6	7.3	C3H	7.0	53.1

Spleen cells from nontransgenic B6D2F₁ mice or mice transgenic (tg) for Cw3, B7, B13, or B2705 (all on B6 or B6D2F₁ background) were used as responders and stimulated *in vitro* with irradiated spleen cells from transgenic B2705 or transgenic Cw3 mice. After 5 days, CTL activity against Cw3 or B27 was measured on ⁵¹Cr-labelled Bc20 fibroblasts (H-2^b, C57Bl/6-derived) transfected with Cw3 or B2705 plus human $\beta 2m$. As a positive control an aliquot of the responder cells was stimulated with normal splenocytes from C3H (H-2^k) mice and the respective CTL tested against 1T.22-6 fibroblasts transfected with K^k (1T.K^k). Data are expressed as per cent specific lysis at an effector:target ratio 100:1. The specific ⁵¹Cr release was calculated as $100 \times (\text{experimental release}) - (\text{spontaneous release}) / (\text{maximum release}) - (\text{spontaneous release})$ (refs 8, 17).

HLA B7 and HLA B13 genes and transgenic mice were produced by E. Lacy and B. Dupont (unpublished), and HLA Cw3 (ref. 24) and HLA B2705 transgenic mice were produced in our laboratory; the B2705 gene was provided by H. Coppin²⁵.

L antigen. Hybrid genes were generated by exchanging the $\alpha 3$ domain of the *H-2K^b* gene with the $\alpha 3$ domain of the HLA A2 gene and transfected into mouse 1T22-6 fibroblasts (H-2^a). Care was taken to use transfectants expressing the same amount of K^b determinants, as measured by cytofluorimetric analysis with K^b-specific monoclonal antibodies. The K^b-specific CTL populations included primary CTL (strain DBA/2 anti-C57Bl/6), most of which were Lyt-2-dependent as judged by inhibition by an anti-Lyt-2 antibody (Fig. 1a). It can be seen from Fig. 1 that

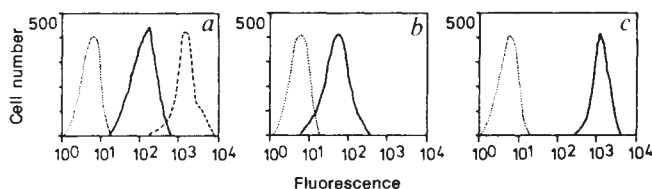
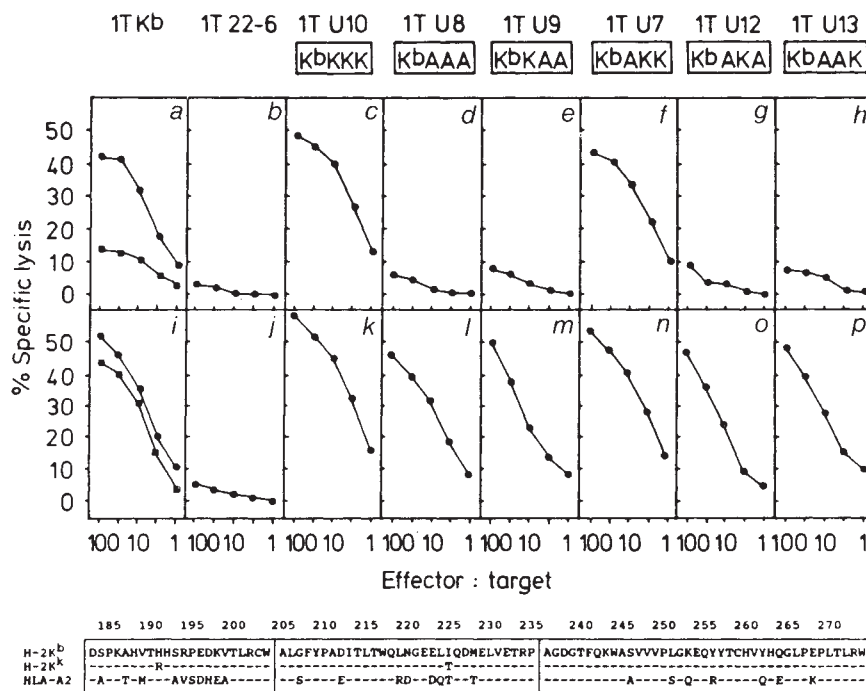


FIG. 2 Expression of HLA-B27 and H-2 class I determinants on peripheral blood lymphocytes of B27/K^d transgenic mice. Peripheral blood lymphocytes from mice transgenic for the HLA *B2705* gene or for the *B27/K^d* hybrid gene containing the $\alpha 1$ and $\alpha 2$ domains of *B2705* and the $\alpha 3$ domain of *H-2K^d* were analysed by cytofluorometry with the monomorphic HLA antibody B9.12.1 (ref. 28). Staining of determinants from *a*, *B2705* mice (dashed line) and *B2705*/human $\beta 2m$ double-transgenic mice (solid line) with B9.12 (ref. 29) and *b*, *B27/K^d* mice with B9.12 (solid line). *c*, Staining of endogenous H-2 with anti-K^b, K^d antibody, 20-8-4S (ref. 17). Dotted lines represent staining with negative control antibody.

METHODS. The *B27/K^d* hybrid gene was constructed by exchange of the exon 4 ($\alpha 3$ domain) of the *B2705* gene²⁵ with the $\alpha 3$ domain of the *H-2K^d* gene²⁶ as before¹⁷. Transgenic mice were produced by microinjection of the *B27/K^d* chimaeric gene or the parental *B2705* gene into fertilized B6 $\times$ DBA/2 eggs²⁴. A human- $\beta 2m$ transgenic mouse (provided by H. Ploegh) was crossed with the B27 transgenic mice. Cytofluorometric analysis of peripheral blood lymphocytes with biotinylated antibodies and fluoresceinated avidin was as described^{8,17}. The fluorescence of 10,000 viable cells was measured in a FACScan cytofluorograph.



exchange of the $\alpha 3$ domain of H-2K^b by the $\alpha 3$ domain of H-2K^k did not affect the lysis by the primary K^b-specific CTL, which is consistent with previous observations that the specificity for alloreactive CTL is determined only by the $\alpha 1$ and $\alpha 2$ domains^{17,18}. When the $\alpha 3$ domain was exchanged by the $\alpha 3$ domain of HLA-A2, no lysis was observed with the primary Lyt-2-dependent CTL but there was strong lysis with the secondary Lyt-2-independent CTL. This indicates that there is possibly a species-barrier in the interaction of the Lyt-2 accessory molecule with the MHC class I target antigen. The $\alpha 3$ domains of H-2K^k and HLA-A2 differ by 18 amino acids (see bottom of Fig. 1). Further intra- $\alpha 3$ exon shuffling between the $\alpha 3$ segments 183-204, 205-233, 234-273 of HLA-A2 and H-2K^k demonstrated that the amino acids located in both segments, 205-233 and 234-273, are important for efficient interaction with the Lyt-2 molecule. These observations are consistent with reports that substitutions of the amino acids 222, 223, 227, 229 and 245 in the $\alpha 3$ domain of HLA ablated the interaction with the human CD8 molecule¹⁴⁻¹⁶. It has been suggested that the CD8 molecule interacts with a loop in the HLA- $\alpha 3$ domain encompassing residues 220-232, and possibly with 233 on the β strand; also that positions 235, 245 and 247 may influence the conformation of the loop and/or the $\alpha 3$ domain¹⁶. In the cellular binding assay used in some of these studies, the human CD8 was able to interact with the mouse H-2K^b and H-2D^p antigens¹⁵, suggesting that the species barrier we observe in the interaction of mouse Lyt-2 with human HLA is unidirectional.

Next we investigated whether modifications of the $\alpha 3$ domain of HLA would result in a more efficient interaction with mouse Lyt-2, thereby facilitating a stronger HLA-specific response of mouse T cells. For this purpose, the $\alpha 3$ domain of the HLA B2705 gene was exchanged with the $\alpha 3$ domain of the murine H-2K^d gene to give transgenic mice with this B27/K^d hybrid gene. The hybrid molecule was expressed on the surface of lymphocytes, as judged by staining with the monomorphic HLA antibody B9.12.1 (Fig. 2b). It can be seen from Fig. 2c that expression was lower than endogenous H-2 class I antigens.

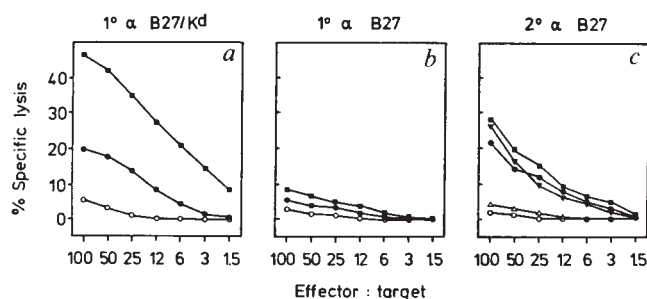


FIG. 3 Generation of primary anti-HLA CTL response against the transgenic B27/K^d hybrid antigen. Normal spleen cells from B6D2F₁ mice were stimulated *in vitro* for 5 days with irradiated spleen cells from transgenic mice expressing the B27/K^d hybrid antigen (a), or stimulated with spleen cells from transgenic mice expressing B2705 plus hβ2m (b). c, Secondary Lyt-2 independent B2705-specific CTL obtained by priming of B6D2F₁ mice with spleen cells from B2705/β2m double-transgenic mice and *in vitro* restimulation in the presence of anti-Lyt-2 antibody (for methods, see Fig. 1). CTL were tested against ⁵¹Cr-labelled target cells: Bc20 fibroblasts (H-2^b), ○; Bc20 transfected with B27/K^d plus hβ2m, ■; Bc20 transfected with B2705 plus hβ2m, ●; human lymphoblastoid cell line BA3874 (A1, 3; B7, 8; C7, —; DR4, 15), △; and HA4135 (A3, 24; B18, 27; C2, —; DR2, 4), ▼. Lymphoblastoid cell lines from D. Kabelitz, Heidelberg.

Expression in B2705 transgenic mice is shown for comparison; this is high in the presence of human β₂ microglobulin (β2m)¹⁹.

When splenocytes from the B27/K^d transgenic mice were used as stimulators for normal T cells derived from C57Bl/6 or (C57Bl/6 × DBA/2)F₁ mice, there was a strong primary CTL response after five days (Fig. 3). The respective CTL lysed target cells transfected with B27/K^d more efficiently than cells transfected with wild-type B2705 (Fig. 3); results were equivalent for eight experiments. There was no appreciable primary response with the same T responder cells stimulated against transgenic spleen cells expressing both B2705 and human β2m (Fig. 3b). Our result is therefore even more striking because the B27/K^d mice show lower levels of expression than the B27/β2m double-transgenic mice (Fig. 2). When we used aliquots of the same responder populations to determine CTL precursor frequencies, about 1/1,300 precursor cells were found to respond to the B27/K^d hybrid molecule. About 1/700 precursor CTL responded to allogeneic H-2^k molecules, whereas only ~1/250,000 responded to the wild-type HLA-B27 molecule. These observations indicate that normal mouse T cells can respond to the xenogeneic HLA-B27 epitopes at about the same frequency as to mouse H-2 alleles, provided that the α3 domain of the B27 molecule is modified in such a way as to permit interaction with the mouse Lyt-2 molecule.

Earlier work indicated that the determinants on class I molecules recognized by CTL depend only on the combined α1 and α2 domains of a particular allele; exchange of the α3 domain had no influence on the CTL determinants^{17,18}. To verify that in this case the K^d-derived α3 domain of the B27/K^d hybrid molecule had not grossly altered the B27 epitopes either, we generated secondary Lyt-2-independent CTL against the B27 wild-type antigen. These CTL could lyse the B27 wild type and the B27/K^d targets, and human B27-positive targets as well (Fig. 3c), indicating that the relevant B27 epitopes are probably identical on these cells.

We conclude that the normal mouse T-cell repertoire contains a large number of T cells with specificity for HLA. Obviously these HLA crossreactive T cells have been positively selected on the thymic H-2 antigens, which is consistent with the HLA and H-2 class I molecules being similar²⁰. But for efficient activation of these HLA crossreactive cells by HLA antigens, the α3 domain should be modified to facilitate the interaction with Lyt-2. The interaction of murine CTL with HLA A2 can be improved when the HLA α3 domain is replaced by the

H-2K^b α3 domain^{21,22}, but in these experiments only a weak primary CTL response against the chimaeric A2/K^b antigen was obtained, probably because transfected cell lines had been used as stimulators. In another report²³ the response of human cells to H-2 antigens was improved in the presence of interleukin-1, which also indicates that appropriate activation of T cells can lead to strong xeno-MHC responses.

Our observation that the HLA B27 response of mice can be strongly enhanced by exchange of the α3 domain could be important for the establishment of animal models for HLA-linked diseases, such as the B27-associated ankylosing spondylitis and Reiter's syndrome. Respective studies with the B27/K^d transgenic mice are in progress. □

Received 19 July; accepted 16 October 1990.

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ACKNOWLEDGEMENTS. We thank H. Ploegh for human β2m-transgenic mice; E. Lacey, B. Dupont and U. Hämmerling for the B7 and B13 genes and transgenic mice; H. Coppin for the B2705 gene; S. Beck for the construction of the B27/K^d transgenic mice; A. Klevenz for technical assistance; M. Simon for help with the limiting dilution analysis, and B. Ermschhaus for preparing the manuscript. Supported by the Deutsche Forschungsgemeinschaft.

ATP sulphurylase activity of the *nodP* and *nodQ* gene products of *Rhizobium meliloti*

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THE symbiotic bacterium *Rhizobium meliloti* stimulates alfalfa (*Medicago sativa* L.) roots to undergo morphogenesis and form nitrogen-fixing nodules. It has been proposed that the bacterial genes *nodABC*, common to all *Rhizobium*, are required for synthesis of an oligosaccharide factor, which is converted to a sulphated form (NodRm-1) by the products of the *R. meliloti*-specific genes *nodH* and *nodQ*¹⁻⁵; NodRm-1 elicits host-specific plant responses². Previously we have shown that the *nodP* gene is homologous to a segment of the *Escherichia coli* genome⁶; when we cloned this *E. coli* fragment we found that it mapped near 59 minutes, corresponding to the *cysDNC* locus. The genes *cysD* and *cysN* encode proteins that catalyse the synthesis of adenosine 5'-phosphosulphate, the first step in the activation of inorganic

sulphate⁷. Here we demonstrate that *nodP* and *nodQ* correspond to *cysD* and *cysN*, and that their proteins have ATP sulphurylase activity both *in vivo* and *in vitro*. We propose that *nodP* and *nodQ* synthesize an activated sulphate that is an intermediate in the formation of the alfalfa-specific sulphated *nodRm-1* factor.

A probe internal to the coding sequence of *nodP* (430-base pair (bp) *SalI*–*SstI* fragment; see Fig. 1) is homologous to genomic DNA of *E. coli*⁶. On the basis of this result, we probed the ordered *E. coli* library of Kohara *et al.*⁸ with the labelled fragment. Two overlapping clones, 451 and 452, hybridized to this probe (Fig. 1). We prepared DNA from these clones and analysed several restriction enzyme digests by using Southern blotting. The same sized fragments from each clone hybridized with the probe (Figs 1 and 2), indicating that the homology is completely contained within the overlapping region. Further analysis of these clones showed that the smallest hybridizing

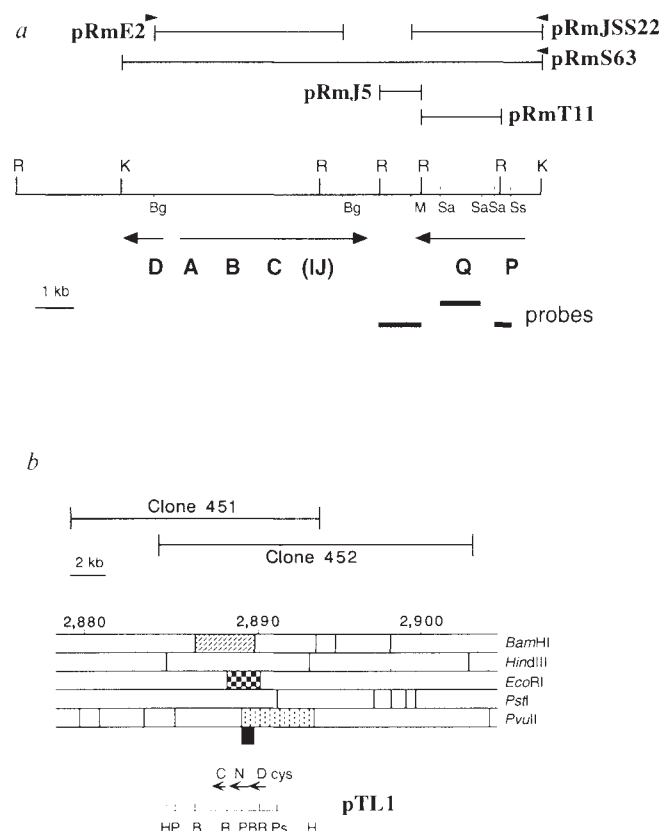


FIG. 1 Map of *nod* genes (shown in bold face) on pSyma of *R. meliloti*^{12,13} (a), and *cys* genes of *E. coli* (b). Restriction enzyme cutting sites are denoted by R, *EcoRI*; K, *KpnI*; H, *HindIII*; P, *PvuII*; B, *BamHI*; Ps, *PstI*. The *R. meliloti* map is incomplete for the restriction enzymes *BglII* (Bg), *MaeI* (M), *SalI* (Sa) and *SstI* (Ss). Arrowheads by plasmid inserts indicate promoter direction; pRmE2 has the *trp* promoter, pRmJSS22 has the T7 RNA polymerase promoter, and pRmS63 has the *lac* promoter. Thick lines represent probes used for hybridization. Arrows indicate size and direction of genes or operons. In the *E. coli* map (b), restriction enzyme fragments (patterned boxes) correspond to those that hybridize to the 430-bp *SalI*–*SstI* *R. meliloti* probe, as shown in Fig. 2. The smallest hybridizing fragment is the *BamHI*–*PvuII* fragment (black rectangle). Map of *E. coli* is adapted from K. E. Rudd and D. A. Benson's digitization of the Kohara *et al.*⁸ library map (b, top), and pTL1 is adapted from ref. 7. In ref. 8, 451 is originally called 1B5, and 452 is originally 6C8.

METHODS. Constructions of pRmE2 (ref. 9), pRmS63 (ref. 6), pRmJ5 (ref. 20), pTL1 (ref. 7), 451 and 452 (ref. 8) have been described. Plasmid pRmT11 is a 2.2-kb *EcoRI* fragment in pBR322 (J. Tu, laboratory stock); pRmJSS22 is a *KpnI*–*MaeI* fragment containing *nodPQ* cloned into the *BamHI* site of the vector pET3 (ref. 21). The fragment was treated with *BamHI* methylase, blunted with Mung Bean nuclease, ligated to *BamHI* linkers, and digested with *BamHI* before ligating to *BamHI*-digested pET3.

band is a 0.9-kilobase (kb) *BamHI*–*PvuII* fragment (Fig. 1b, and data not shown), which maps to ~2,890 kb on the Kohara map, or 59 min on the *E. coli* chromosome⁹. Of the genes mapping near this position, the *cysDNC* region, cloned by Leyh *et al.*⁷, has the same restriction enzyme map for all of the enzymes that the two maps have in common (although the size estimates of the map of ref. 7 are consistently larger). Thus we have located the *cysDNC* region precisely on the *E. coli* genomic restriction map, between *recA* and *iap* consistent with information provided by G. D. Markham (personal communication).

The *E. coli* genes *cysD* and *cysN* code for proteins that together have ATP sulphurylase activity (catalysing the reaction $\text{ATP} + \text{SO}_4^{2-} \rightarrow \text{APS} + \text{PP}_i$, where APS is adenosine 5'-phosphosulphate and PP_i is inorganic pyrophosphate), whereas *cysC* encodes APS kinase. In *E. coli* these genes create an activated sulphate group in the form of PAPS (3'-phosphoadenosine 5'-phosphosulphate), which is reduced and used in cysteine biosynthesis¹⁰. To show that *nodP* and possibly *nodQ* are functionally equivalent to these *cys* genes, we transformed plasmids containing different sets of *nod* genes into *E. coli* cysteine auxotrophs with mutations in *cysD*, *cysN* or *cysC*. The results shown in Table 1 indicate that *nod* genes can substitute for ATP sulphurylase (CysD and CysN) as well as APS kinase activity (CysC). Plasmid pRmS63 complements all three auxotrophs to cysteine prototrophy, whereas pRmJSS22, in the presence of small amounts of T7 polymerase, only complements TSL3, the *cysD* mutant. DM62, the *cysN* mutant, has neither ATP sulphurylase activity nor APS kinase activity, because the mutation is polar⁷. Clones containing only the common *nod* genes (pRmE2), partial coding regions (pRmJ5 and pRmT11), or no usable *E. coli* promoter (pRmJSS23) do not complement the auxotrophs (Table 1 and Fig. 1). In addition, the 1.15-kb *SalI* fragment containing sequences internal to *nodQ* hybridizes to the same *E. coli* fragments as the *nodP* probe in Fig. 1 (data not shown). The differences between this result and our previous report of no homology between *nodQ* and *E. coli*⁶ is probably due to our use here of a probe with more 5' *nodQ* sequences. This evidence leads us to speculate that *nodP* and *nodQ* together encode ATP sulphurylase. There would be sufficient room for a *cysC* (APS

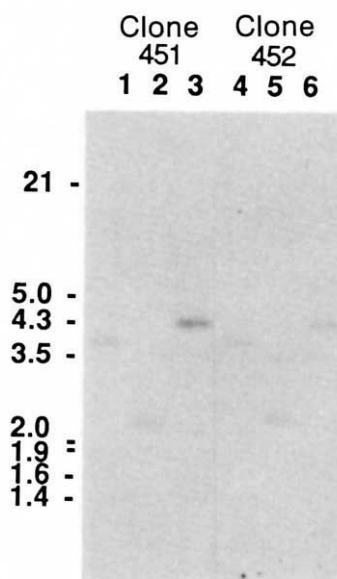


FIG. 2 Southern blot of clones 451 and 452, probed with *nodPQ* sequences. DNA was digested with *BamHI* (lanes 1 and 4), *EcoRI* (lanes 2 and 5) or *PvuII* (lanes 3 and 6). Migration of molecular weight standards is shown on the left (kb).

METHODS. DNA was isolated from lambda phage clones²², and blotted onto Magnagraph nylon membranes as before^{23,24}. The 430-bp *SalI*–*SstI* fragment internal to *nodP* was labelled using the random hexamer primer technique²⁵. Nylon membranes were hybridized and washed as described⁶.

kinase) homologue downstream of *nodQ* and before the next known *nod* gene. However, we could not detect homology between *E. coli* and *R. meliloti* DNA on a Southern blot of pTL1 using the pRmJ5 insert (Fig. 1) as a probe (data not shown). We used this same blot to confirm the homology between pTL1 and pRmT11 (data not shown). This does not rule out the presence of a functional homologue to *cysC* on pRmS63; it may indicate that the sequence is somewhat more diverged.

The confirmation of a function for nodulation genes can proceed by demonstration of *in vitro* function of *nod* genes or their proteins, or by biochemical analysis of mutant phenotypes. We have established ATP sulphurylation activity for the products of cloned *nodPQ* genes. We prepared crude cell extracts from *E. coli* strains expressing CysD and CysN, NodP and

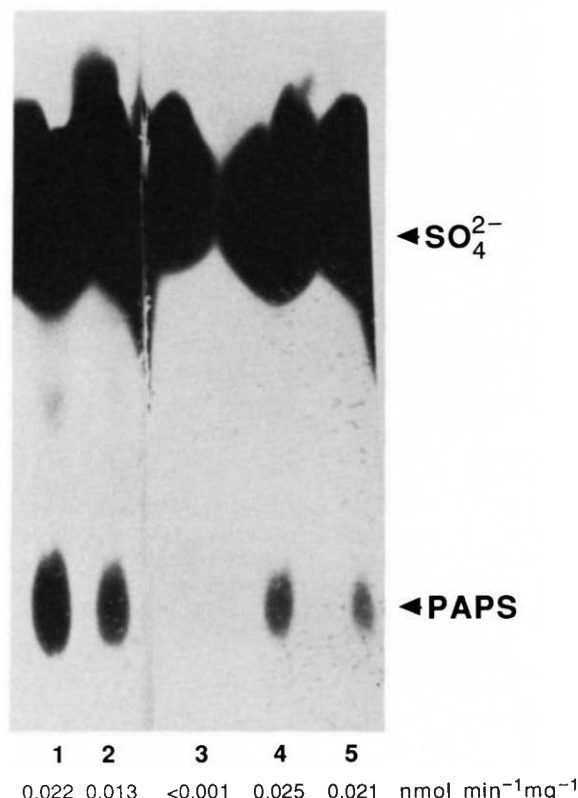


FIG. 3 Thin-layer chromatography of products from coupled ATP sulphurylation assay. Assay in lane 1 was carried out with extract from Rm1021/pRmT5; lane 2, Rm1021; lane 3, BL21(DE3)/pRmJSS23; lane 4, BL21(DE3)/pRmJSS22; lane 5, BL21(DE3)/pTL1. pRmT5 (ref. 12) contains the host-specific *nod* region of *R. meliloti*, including *nodPQ*; BL21(DE3) (ref. 26) is an *E. coli* strain containing an Isopropyl β -D thio galactopyranoside (IPTG)-inducible T7 RNA polymerase.

METHODS. Procedures adapted from ref. 7. *R. meliloti* extracts were prepared from 5 ml cells grown in M9 sucrose to an absorbance of one at 600 nm. Cells were spun down and washed in 1 ml 20 mM Tris-HCl, pH 8, resuspended in 100 μ l buffer B (ref. 7) and sonicated. *E. coli* extracts were prepared from cells grown in Luria-Bertani: Super Broth to an absorbance of one at 600 nm + 1% glycerol. T7 RNA polymerase was induced for 10 min with 400 μ M IPTG, and pT7-specific proteins were amplified with 200 μ g ml⁻¹ rifampicin for 30 min. Cells were washed in a half volume of 20 mM Tris, pH 8, resuspended in 0.1 volumes of buffer B, and broken either by sonication (BL21(DE3)/pTL1), or French Press at 13,000 p.s.i. (BL21(DE3)/pRmJSS22 and 23). French press extracts were then treated with 0.25 volumes of 5% streptomycin sulphate. All extracts were spun for 15 min in a microfuge to remove cell debris. Supernatants (3 μ l) were added to the reaction mix, which contained ATP, Na₂³⁵SO₄, APS kinase (kindly provided by C. Satishchandran and G. D. Markham²⁷), inorganic pyrophosphatase and salts⁷. Products of the reaction were chromatographed on Polyethyleneimine-fluor cellulose as previously described, except that they were developed with 1.5 M LiCl. Counts per min were measured using the AMBIS two-dimensional radioactivity detector.

TABLE 1 Complementation of *E. coli* cysteine auxotrophs with *R. meliloti* genes*

Plasmids	JM81A (<i>cysC92</i>)	Strains ⁷ : DM62 (<i>cysN96:kan</i>)	TSL3 (<i>cysD91</i>)
pUC18 (ref. 17)	—	—	—
pRmS63	+	+	+
pRmE2	—	—	—
pRmJ5	—	—	—
pRmT11	—	—	—
pRmJSS22	—	—	—
pRmJSS23†	—	—	—
pRmJSS22 + pGP1-2‡	—	ND	+
pRmJSS23 + pGP1-2	—	ND	—

* Strains were tested on M9 glucose plus ampicillin (50 μ g ml⁻¹) and supplemented with leucine, proline and thiamine; (+) indicates growth without cysteine supplementation, (—) indicates no growth without cysteine; ND, not determined.

† Plasmid pRmJSS23 contains the same insert as pRmJSS22 in pET3 but in opposite orientation with respect to the T7 promoter.

‡ Plasmid pGP1-2 (ref. 18) with T7 RNA polymerase under the control of a heat-shock promoter was selected with kanamycin at 25 μ g ml⁻¹. Strains with this plasmid were grown at 30 °C.

NodQ, or neither pair, and measured ATP sulphurylation activity in these extracts (Fig. 3). This assay couples ATP sulphurylation activity with excess APS kinase and inorganic pyrophosphatase, as the $K_{eq} = 10^{-9}$ for APS and PP_i production⁷. Thus PAPS production is monitored and used as a measure of APS production. We found that *E. coli* extracts containing NodP and NodQ have ATP sulphurylation activity *in vitro*. In addition, extracts from wild-type *R. meliloti* strains also have ATP sulphurylation activity (Fig. 3).

Faucher *et al.*⁴ have proposed that *nodABC* gene products are involved in synthesis of a root hair deformation factor (HadV) effective on vetch, and that conversion of this to an alfalfa-specific factor, HadA, requires the action of *nodH* and *nodQ*. Lerouge *et al.*^{5,11} have proposed that HadV is an *N*-acyl-derivatized tetramer of *N*-acetylglucosamine, and that the alfalfa-specific form HadA is NodRm-1, which is sulphated on the reducing end *N*-acetylglucosamine residue. Synthesis of normal levels of NodRm-1 factor requires *nodH* and *nodQ*. The production of factors by *nodP* mutants has not been studied, but *nodP* mutants show a delay in nodulation¹², as do *nodQ* mutants^{12,13}. This is consistent with our finding that *nodP* and *nodQ* encode a sulphate-activating enzyme. Because *nodP* and *nodQ* have overlapping reading frames^{6,14} and because CysD and CysN act together to form a single enzyme, we would predict that *nodP* mutants would act like *nodQ* mutants with respect to factor production.

We propose then that *nodP* and *nodQ* are involved in creating an activated form of sulphate (APS), which is then transferred to a NodRm-1 precursor, possibly by NodH¹¹. As yet, we do not know whether APS or PAPS is the sulphate donor in NodRm-1 synthesis; PAPS is known to act as sulphate donor in other systems^{10,15}. We also do not yet know the role of the second locus of *nodP* and *nodQ* in *R. meliloti* that we have previously reported⁶, but we speculate that the different copies may be under the control of different regulatory networks. For example, the copy located in the *nod* gene cluster may be co-regulated with other nodulation genes involved in NodRm-1 factor production¹⁶, whereas the second copy may be regulated with respect to cellular cysteine levels. Further analysis of the function and regulation of the second *nodPQ* copy is in progress. □

Received 28 August; accepted 12 October 1990.

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ACKNOWLEDGEMENTS. This work was supported by the Department of Energy (S.R.L.), by a training grant in Cell and Molecular Biology from the NIH to Stanford University and by an ARCS fellowship (J.S.S.). We thank G. D. Markham and C. Satishchandra for *E. coli* strains, plasmids and APS kinase; T. Leyh and G. D. Markham for communicating data before publication; M. McCann for an amplified Kohara phage library; R. Kornberg and members of his laboratory, and M. Ray for use of the AMBIS 20 radioactivity detector; W. Margolin and F. W. Studier for T7 RNA polymerase expression systems; D. W. Ehrhardt and E. M. Atkinson for discussion; J. Swanson for *R. melioli* strains and plasmids; B. T. White and R. F. Fisher for reviewing the manuscript, and A. Bloom for her help in its preparation.

Point mutation in the exoplasmic domain of the erythropoietin receptor resulting in hormone-independent activation and tumorigenicity

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THE receptors for erythropoietin and other cytokines constitute a new superfamily¹⁻⁴. They have no tyrosine-kinase or other enzyme motif and their signal-transducing mechanism is unclear. Here we describe two classes of activating mutations in the erythropoietin receptor (EPOR). A single point mutation in the exoplasmic domain enables it to induce hormone-independent cell growth and tumorigenesis after expression in nontumorigenic, interleukin-3-dependent haematopoietic cells. A C-terminal truncation in the cytoplasmic domain of the EPOR renders the receptor hyper-responsive to erythropoietin, but is insufficient to induce hormone-independent growth or tumorigenicity. The activating point mutation retards intracellular transport and turnover of the receptor. These alterations in metabolism and tumorigenicity caused by the EPOR with activating point mutations are similar to those observed in erythropoietin-independent activation of the wild type EPOR by association with gp55, the Friend spleen focus-forming virus glycoprotein^{5,6}.

Erythropoietin is a glycoprotein hormone of relative molecular mass 34,000 (M_r 34K) that induces proliferation and differentiation of erythroid progenitor cells. Following the cloning of the murine EPOR complementary DNA⁷, we developed

a cell-culture system to study the proliferative action of the receptor. Expression of the EPOR cDNA allows interleukin-3 (IL-3)-dependent haematopoietic Ba/F3 cells to grow in the presence of erythropoietin^{5,6}. Similarly, Ba/F3 cells can be switched to IL-2-dependent growth by expression of the IL-2 receptor β -chain (IL-2R β)⁸. As regions of the cytoplasmic domain of the IL-2R β , IL-3 receptor, and EPOR are similar¹⁻³, these receptors may share similar signal transducing pathway(s).

Some receptors containing tyrosine-kinase domains can be constitutively activated by point mutations and deletions. Such mutations are frequently found in retroviral oncogenes (for example, *v-fms* and *v-neu*), whose origins are normal cellular genes. But no constitutively activated mutants of any member of the cytokine receptor family have been detected to date. Thus, we have tried to isolate mutant EPORs from Ba/F3 cells infected with retroviruses expressing the receptor cDNA⁵. As the virus replicates in the packaging cells^{9,10}, spontaneous mutations in the EPOR gene may occur, just as in the creation of oncogenes *in vivo*.

After selection by gradual decreases in the concentration of erythropoietin in the medium, we obtained two classes of mutant

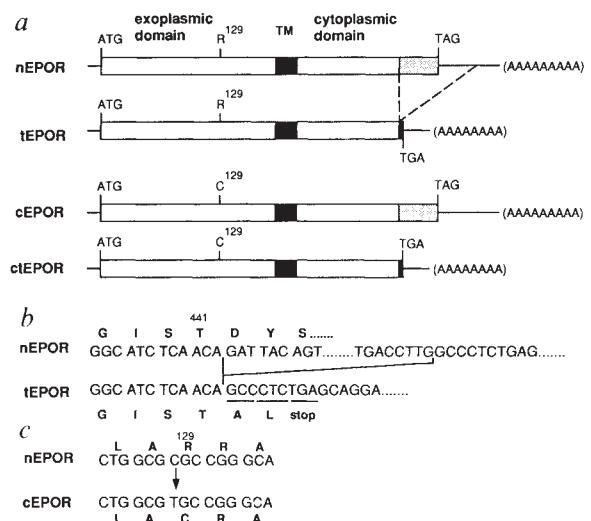


FIG. 1 a, Comparison of wild-type and mutant EPOR cDNAs. b, Deletion mutation in the mutant cEPOR cDNA. Numbers of the amino acids, codon positions from the predicted N-terminus⁷. The transmembrane domain (TM), initiation (ATG) and termination (TAG or TGA) codons are indicated. Single lines, non-translated regions of the messenger RNA.

METHODS. Isolation of mutant cell lines: A retroviral vector containing the wild-type EPOR cDNA (pSF-ER) was packaged and amplified in a mixture of helper cell lines, psi-cre and psi-crip^{5,10}. Infected Ba/F3 cells (1×10^7) were selected by incubating cells for 7 days in medium containing a physiological concentration of erythropoietin (0.1 U ml⁻¹ or 10 pM). All clones eventually isolated in this selection medium expressed EPOR. Cells were further selected by incubating cells in 0.01 U ml⁻¹ erythropoietin for 14 days. At this concentration, cells expressing the wild-type receptor cannot grow. All 12 clones obtained in this selection medium contained tEPOR, judged by lack of reactivity with an anti-C-terminal antibody⁶ (data not shown) and by molecular size (Fig. 2a). One of these clones, cEPOR, was found to grow in the absence of any growth factor. Another factor-independent cell clone, cEPOR, was isolated by culturing Ba/F3 cells infected with the same virus sample in the absence of erythropoietin using a similar procedure (Fig. 2a). Cloning and sequencing of the mutant EPOR cDNA: Integrated full-length EPOR cDNA was directly amplified from genomic DNA of wild type and cEPOR cells by using polymerase chain reaction (PCR)¹¹ primers N1 (5'-AAGGTACCTGAAGCT-AGGGTGCATCA-3'; bases -18-1) and C1 (5'-GGGAATTCGGTGGAGTCTAGGAGCAG-3'; bases 1,561-1,542), then cloned into M13mp18, M13mp19 and pXM using *EcoRI* and *KpnI* cloning sites. For tEPOR and cEPOR, two partially overlapping cDNA fragments that covered the full-length cDNA were obtained by using two PCR primer sets: N1 and P11 (5'-GCAGAGTCCGGCGGTGGG-3'; bases 859-842), and also P3 (5'-GACCAACCCAC-ATCCGATATG-3'; bases 546-565) and PB2b (5'-AAGCTTAACATTGCAAGGCT-3'; bases 1,654-1,638). The former fragments were cloned into M13 vectors using *KpnI* and *NheI* sites, and the latter ones cloned into the vector as two separate fragments after digestion with *HindIII*. Single-stranded DNA was obtained and sequenced using synthetic oligonucleotide primers according to Sanger *et al.*¹² Constructs of tEPOR and cEPOR in pXM were made from pXM-nEPOR or pXM-cEPOR cDNA by replacing the *HindIII-EcoRI* fragment (corresponding to the C-terminal region of EPOR cDNA) with the *HindIII-EcoRI* fragment of the tEPOR cDNA cloned in M13 double-stranded DNA.

TABLE 1 Tumorigenicity of Ba/F3 clones expressing EPOR

cDNA	Tumours in mice injected with clones derived from:	
	Plasmid (pXM) transfection	Retroviral infections
mock	0/5	N.D.
nEPOR	0/4	0/4
tEPOR	N.D.	0/6
cEPOR	6/6	6/6
nEPOR-gp55	N.D.	6/8

Wild-type and mutant EPOR-expressing Ba/F3 cells (retrovirus infectants isolated as described in Fig. 1 and pXM transfectants as described in Fig. 3) were injected into syngeneic Balb/c mice <12 weeks old. Mice received 0.5 Gy (500 rads) of γ -irradiation 24 h before cell challenge. Cells ($3-5 \times 10^6$ per mouse) were washed with serum-free medium, resuspended in Hanks' balanced salt solution, and injected into mice subcutaneously. Mice were observed for 2-3 months for signs of palpable or visible tumours at the site of injection. Tumorigenic cell lines gave rise to visible masses with a short latency after injection (2-3 weeks) which was nonregressing and malignant. Representative mice injected with nontumorigenic cell lines were killed and autopsies performed to ensure tumours had not developed.

N.D., not determined.

cell lines: one is 3-5 times more responsive to erythropoietin than are cells expressing the wild-type receptor (nEPOR) but cannot grow in the absence of growth factor. This cell line has a C-terminal truncation in the EPOR (tEPOR). The other mutants grow in the absence of any haematopoietic growth factors. We isolated two such constitutive receptors; one is normal size (cEPOR) and the other is truncated in the C-terminus (ctEPOR) (Fig. 1 legend).

Mutation sites in the integrated receptor cDNA were identified as detailed in Fig. 1. In tEPOR, a 193 base-pair (bp) deletion spanning the 3' coding and noncoding region (from $^{1,424}G$ to $^{1,616}G$) resulted in replacing the normal C-terminal 42 amino acids with alanine (A) and leucine (L) (Fig. 1b). In cEPOR there was one point mutation—a transition from C to T at nucleotide 484 causing one substitution in the exoplasmic

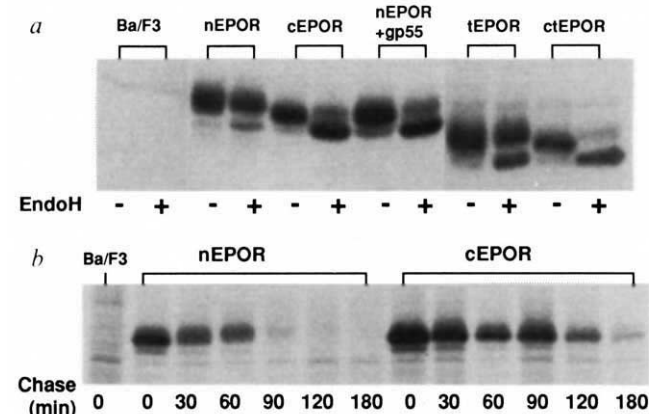


FIG. 2 Expression of EPOR. *a*, Parental Ba/F3 cells were cultured in 10% WEHI-conditioned medium as a source of IL-3 (ref. 6). The nEPOR cells and tEPOR cells were cultured in 0.1 U ml^{-1} and 0.01 U ml^{-1} EPO, respectively. The cEPOR, ctEPOR and nEPOR-gp55 cells^{5,6} were cultured in normal RPMI medium. Cells were retrovirus infectants isolated as described in Fig. 1. Membranes from cells were digested with or without EndoH, separated by SDS-PAGE in 7.5% polyacrylamide gels, and immunoblotted with an antibody against a peptide corresponding to the N-terminal 14 amino acids of the EPOR as described previously^{5,6}. Similar results were obtained from plasmid pXM transfectants (data not shown). *b*, Cells expressing nEPOR or cEPOR (retroviral transfectants) were metabolically labelled with [^{35}S]methionine for 30 min and chased for the indicated periods. Samples were immunoprecipitated with the above antibody against the EPOR, and subjected to 7.5% SDS-PAGE and fluorography⁶.

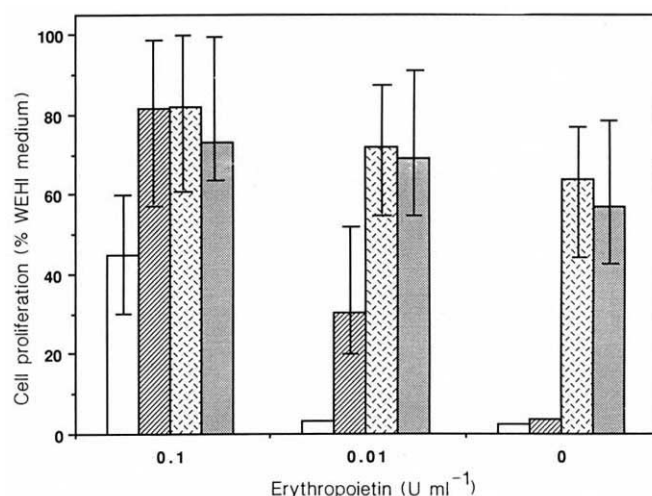


FIG. 3 Growth of Ba/F3-EPOR transfectants in different concentrations of erythropoietin. Open boxes, nEPOR; diagonal lines, tEPOR; hatched, cEPOR; grey, ctEPOR. Wild-type or mutant EPOR cDNAs were subcloned into pXM as described in Fig. 1. Ba/F3 cells (1×10^7) were transfected by electroporation with 20 μg of the plasmids linearized with *Xba*I. Cells were further cultured in medium supplemented with 10% WEHI-conditioned medium. After 24 h further incubation, cells were washed and cultured in 0.25 U ml^{-1} of erythropoietin for 5 days to select cells expressing an EPOR. Individual clones were isolated by limited dilution. These clones were expanded in 10% WEHI-conditioned medium and were tested for growth-factor sensitivity. Cells (1,000 per well) were cultured in the presence of 10% WEHI-conditioned medium, 0.1 U ml^{-1} erythropoietin, 0.01 U ml^{-1} erythropoietin, or no added growth factor for 3 days. The number of living cells was estimated by colorimetric MTT assay⁵. Growth of cells from each clone in the presence of differing amounts of erythropoietin or with no added growth factor was normalized to growth in the presence of 10% WEHI-conditioned medium. The bars represent an average of all clones of each type tested (nEPOR, 7 clones; tEPOR, 8 clones; cEPOR 11 clones; ctEPOR, 7 clones) and error bars define the range of proliferation values (maximum and minimum values).

domain, from arginine (R) to cysteine (C) at codon 129 of the predicted N-terminus (after signal sequence processing)⁷ (Fig. 1c). In ctEPOR, exactly the same point mutation as in cEPOR and the same deletion as in tEPOR were found.

To show that these mutations are responsible for the mutant phenotype, the isolated mutant EPOR cDNAs were subcloned into the mammalian expression vector pXM (ref. 7) and introduced into Ba/F3 cells (Fig. 3). Transfection with tEPOR cDNA resulted in cell lines able to grow in one-tenth the physiological concentration of erythropoietin ($0.01 \text{ unit ml}^{-1}$), but still unable to grow in the absence of erythropoietin. Only transfection of cEPOR and ctEPOR cDNAs generated factor-independent cell lines. Thus the R $\rightarrow$ C point mutation is enough to induce factor-independent growth, and deletion of the C-terminal 42 amino acids enhances the sensitivity to erythropoietin for growth.

Factor-independent Ba/F3 cells can also form tumours in syngeneic mice (Table 1). Parental Ba/F3 cells, or erythropoietin-dependent transformants (nEPOR cells or tEPOR cells) were not tumorigenic, but hormone-independent cEPOR cells and nEPOR-gp55 cells (created by infection of nEPOR cells with SFFV⁵) generate tumours in mice. This suggests that constitutively activated receptors have tumorigenic potential.

The point mutation in the exoplasmic domain affects receptor metabolism. As described previously⁶, wild-type EPOR is synthesized as a major 64K endoglycosidase H (EndoH)-sensitive species and a minor 62K unglycosylated form. The 64K form is processed to a 66K mature EndoH-resistant form, a fraction of which is on the cell surface. All three forms are degraded very quickly, with a half-life of about 40 min (Fig. 2b). The tEPOR has similar processing and half-life (data not shown). At steady-state, 60-80% of the nEPOR and tEPOR

polypeptides are the EndoH-resistant mature species. By contrast, cEPOR and ctEPOR cells contain very little EndoH-resistant receptor (Fig. 2a). Pulse-chase experiments show little processing of the cEPOR to a 66K, EndoH-resistant species, and the half-life of the cEPOR was longer than that of the nEPOR (Fig. 2b). Interestingly, such retention of the receptor polypeptide in the endoplasmic reticulum was also seen in the hormone-independent cell line expressing nEPOR and gp55 (nEPOR-gp55) (Fig. 2a and ref. 6). These data suggest that a similar activating conformational change of the EPOR can occur by a point mutation or through binding of gp55. However, neither the codon-129 mutation nor association with gp55 affects ligand-binding affinity of the cell surface receptor (data not shown).

Activation of the EPOR by a point mutation in the exoplasmic domain is quite reminiscent of that of *c-fms*, the receptor for macrophage colony stimulating factor (M-CSF/CSF-1)¹³⁻¹⁶. A single point mutation in the exoplasmic domain activates the tyrosine kinase of *c-fms*, perturbs receptor transport and turnover, does not affect ligand binding and results in transformation. Also, modifications at the C-terminal region of *c-fms* increase ligand responsiveness but are themselves insufficient to induce transformation.

Recent studies suggest that autocrine activation of the cytokine receptors are involved in leukaemogenesis¹⁷⁻²⁰. Our

data raise the possibility that mutational activation of the EPOR or other cytokine receptors could be a mechanism for overriding normal hormonal control of proliferation of haematopoietic cells. □

Received 17 August; accepted 12 October 1990.

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ACKNOWLEDGEMENTS. We thank A. D. D'Andrea for antibodies against EPOR and also retroviral supernatant containing EPOR cDNA. We also thank Y. K. Yoshimura for technical assistance, E. Kaji for criticisms of the text, and M. Boucher for help with the manuscript. Supported by the Nakatomi Health Science Foundation (Saga, Japan) (A.Y.), the NIH/NIA (G.L.) and the NIH (H.F.L.).

Consecutive inactivation of both alleles of the *pim-1* proto-oncogene by homologous recombination in embryonic stem cells

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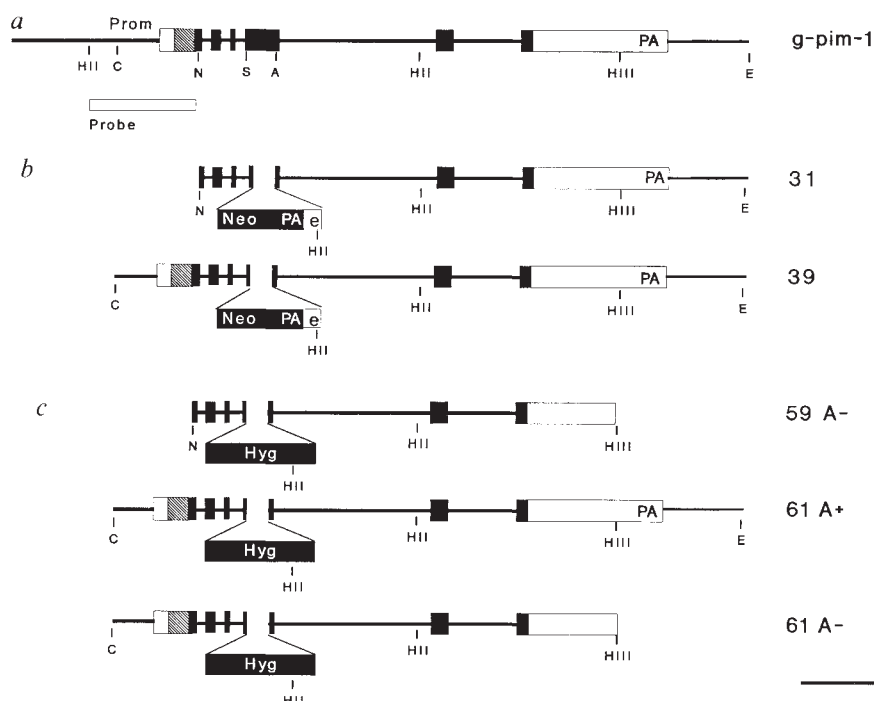
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SPECIFIC genes can be inactivated or mutated in the mouse germ line¹. The phenotypic consequences of the mutation can provide pivotal information on the function of the gene in development and maintenance of the mammalian organism. The procedure entails homologous recombination in embryonic stem cells, which, on fusion to recipient blastocysts, give rise to chimaeric mice that can transmit the mutant gene to their offspring. Inbreeding can then yield mice carrying the mutation in both alleles allowing the phenotypic analysis of recessive mutations. In addition to mice lacking a particular gene function, cell lines carrying null alleles of normally expressed genes can be instrumental in assessing the function of the gene. These cell lines can either be obtained from homozygous animals or, should the mutation be lethal early in embryonic development, be generated by consecutive inactivation of both alleles by homologous recombination in cultured cells. Here we illustrate the feasibility of this latter approach by the efficient consecutive inactivation of both alleles of the *pim-1* proto-oncogene in embryonic stem cells.

The *pim-1* proto-oncogene encodes a cytoplasmic protein serine/threonine kinase (C. J. M. Saris, J. Domen and A. Berns, manuscript submitted). Expression is high in haematopoietic tissues^{2,3}, testis⁴ and ovaries (J. Domen *et al.*, unpublished results). Transgenic mice that overproduce Pim-1 protein in their

lymphoid tissues are predisposed to T-cell lymphomagenesis^{5,6}. Although the oncogenic potential of *pim-1* is clearly established, little is known about the normal function of *pim-1*. To obtain cells that completely lack Pim-1 protein, we have inactivated both alleles of the *pim-1* gene by homologous recombination in embryonic stem (ES) cells. As the *pim-1* gene is highly expressed in ES cells (P. Laird *et al.*, unpublished results), effects of *pim-1* ablation might already become apparent during *in vitro* propagation and differentiation of ES cells. Disruption of the *pim-1* gene was achieved by electroporating ES cells with *pim-1* fusion constructs that conferred resistance to G418 or hygromycin B. Double crossing-over at the *pim-1* locus will integrate the marker sequences into the fourth exon of the *pim-1* gene, thereby deleting a large portion of the sequence encoding the kinase domain⁷ (Fig. 1). The *pim-1*-minus ES cell lines were generated by a two-step selection procedure: first, single knock-outs were obtained by integration of the *neo* gene in one of the *pim-1* alleles; then the remaining wild-type *pim-1* copy was disrupted by integration of the *hyg* sequence. To select for homologous recombinants at the *pim-1* locus among the far more frequent random integrations, expression of the marker genes was made dependent on the acquisition of transcriptional and translational start signals from host DNA. This was achieved by depriving the coding sequences of the bacterial *neo* and *hyg* genes of their own translational start codons and fusing their 5' end in frame to part of the *pim-1* coding sequence. Only rarely would random integration be expected to result in the functional expression of the resistance markers. But homologous recombination will provide the selectable genes with a functional promoter and translation initiation site, leading to the synthesis of Pim-Neo or Pim-Hyg fusion proteins, respectively (Fig. 1). There are two requirements for successful application of this selection scheme. First, the target gene in the recipient cell must already be active or inducible on homologous recombination with the targeting construct. This requirement is fulfilled in the case of the *pim-1* gene. Second, the fusion proteins must still be able to confer resistance to G418 or hygromycin B, respectively. For *pim-1*, this was verified by expressing identical fusion constructs in *Escherichia coli* using a *pim-1* complementary DNA fragment (gift of C. Saris). A construct containing a cDNA fragment encoding the first 98 *pim-1* amino acids fused to the fifth codon of the neomycin phosphotransferase gene gave high resistance

FIG. 1 *a*, Genomic structure of the *pim-1* gene⁷. Two translational initiation codons are used: an ATG at the end of exon 1 giving a protein of relative molecular mass 34,000 (M_r , 34K) (coding region in black); an in frame CTG, which extends the coding region (hatched region in exon 1) to give a 44K protein (C. J. M. Saris, J. Domen and A. Berns, manuscript submitted). A, *AccI*; C, *Clal*; E, *EcoRI*; HII, *HindII*; HIII, *HindIII*; N, *NarI*; S, *SacI*. DNA targeting constructs contain *neo* (*b*) or *hyg* (*c*) inserted between the *SacI* and *AccI* sites of exon 4, deleting 296 *pim-1* nucleotides. At the *SacI* site, the 98th *pim-1* codon (counted from the ATG) is fused in frame by two extra codons (Ser, Leu) to the 5th *neo* codon (pKM109-90 was used¹³). The 3'-end of *neo* including the polyadenylation signal was derived from pMC1Neo poly(A)¹⁴. The cassette also contains the PyF101 enhancer (e in Fig. 1*b*, from pΔMo + PyF101, ref. 15), to ensure expression of *neo* on homologous recombination in ES cells. As *pim-1* is highly expressed in ES cells, the enhancer is probably superfluous. In the *hyg* construct, the *pim-1* sequence is fused through one extra codon (Leu) to the second *hyg* codon. Convenient restriction sites flanking the *hyg* coding sequence (derived from pLG89, ref. 16 and pIT219, ref. 17) were generated using the polymerase chain reaction (PCR). No polyadenylation signal was added and the *pim-1* polyadenylation signal was excluded from the targeting construct 59A⁻, which may provide additional selection for homologous recombination. As a control for expression of the fusion constructs in ES cells, the targeting constructs were extended at their 5' end to include the *pim-1* promoter and translational initiation codons. The *hyg* construct was also extended at its 3' end to include the *pim-1* polyadenylation signal. The targeting constructs were excised from the cloning vectors using endogenous *pim-1* sites, *EcoRI* (31) and *HindIII* (59) on one side and



a polylinker site, *EcoRV*, on the other. The 5' end of the targeting constructs contains, therefore, five extra non-homologous nucleotides adjacent to the *pim-1* *NarI* site. The control constructs were excised by *Clal*-*EcoRI* or *Clal*-*HindIII* digests. All fragments were separated from vector sequences by gel electrophoresis and purified by electro-elution. Bar, 1 kilobase pair.

to kanamycin. Fusion of the same *pim-1* sequence to the second codon of the *hyg* sequence gave moderate resistance to hygromycin B, whereas fusion to the fourth codon did not. To verify functional expression of the fusion constructs in ES cells, the targeting constructs were extended at their 5' end to include the *pim-1* promoter and translational start sites.

Electroporation of ES cells with the *pim-neo* constructs 39 and 31 (Fig. 1*b*) revealed that the absence of a promoter and a translational start codon reduced the number of G418-resistant colonies 20-fold (Table 1*a*). Of 40 G418-resistant colonies obtained with the promoter/AUG-minus construct, 34 (85%) underwent homologous recombination at one of the *pim-1* alleles (see Fig. 2*a* for DNA analysis). Using the *hyg* gene, 62% (26 out of 42 tested) of the colonies contained the *hyg* sequence integrated in one of the *pim-1* alleles (Table 1*b* and Fig. 2*b*). Although the percentage of homologous recombinants among the resistant colonies obtained with the *pim-1-neo* and *pim-1-hyg* targeting constructs was very similar (85% versus 62%), the absolute number of hygromycin B-resistant colonies was significantly lower than the number of G418-resistant colonies (compare Table 1, construct 31 and 59A⁻). However, the *neo* and *hyg* constructs differ in too many aspects to allow an unequivocal explanation for this difference. The ES cells targeted with the *pim-hyg* construct showed normal morphology and growth characteristics.

To obtain a double knock-out of the *pim-1* gene, an ES cell clone containing the *neo* marker in one of the *pim-1* copies was expanded and electroporated with the *hyg* targeting construct. Sixty-two per cent of the colonies resistant to hygromycin B (21 out of 34 tested) had undergone homologous recombination (Table 1*c* and Fig. 2*c*). In 6 of these colonies, the *hyg* sequence had replaced the *neo* sequence; the remaining 15 carried the *neo* marker in one *pim-1* allele and the *hyg* marker in the other allele. In one clone, the wild-type *pim-1* restriction fragment was found in conjunction with both *neo*- and *hyg*-modified

pim-1 fragments (Fig. 2*c*, lane 6), suggesting that this clone was trisomic for the *pim-1* locus or that flanking sequences had been transferred from the endogenous *pim-1* gene to the targeting construct, which then integrated elsewhere⁸. Apparently, either of these events occur only at low frequency. The efficiencies of *pim-1* gene targeting in the single and double knock-out experiments were similar; no selection against double *pim-1*-knock-outs in ES cells was found. Colonies grew well and no differences in ES cell morphology were observed.

ES cell lines carrying two wild-type *pim-1* alleles, a single *pim-1* knock-out or a double *pim-1* knock-out, were allowed to

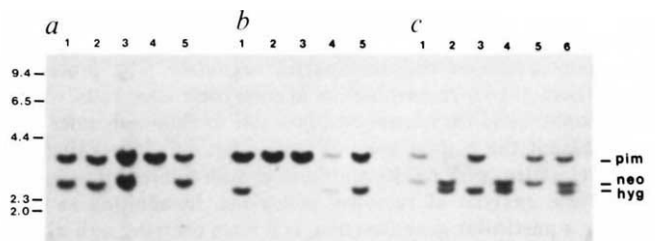


FIG. 2 Genetic modification of the *pim-1* locus by homologous recombination. DNAs of individual clones resistant to G418 or hygromycin B were digested with *HindII* and analysed by Southern hybridization using the genomic *HindII*-*BstXI* *pim-1* fragment (Fig. 1) as a probe. The non-modified *pim-1* locus gives a band of 3,565 base pairs (bp) (*pim*); integration of *neo* gives a 2,663 bp band (*neo*), integration of *hyg* gives a 2,453 bp band (*hyg*). *a*, Disruption of one *pim-1* allele by *neo* insertion (lanes 1, 2, 3, 5). Lane 4 represents a clone in which *pim-1* is not modified. *b*, Disruption of one *pim-1* allele by *hyg* insertion (lanes 1, 4, 5). Lanes 2 and 3 represent clones in which *pim-1* is not modified. *c*, Disruption of both *pim-1* alleles by *neo* and *hyg* insertion (lanes 2, 4). Lane 3: *hyg* has replaced *neo*; Lanes 1 and 5: No targeting by *hyg* has occurred. Lane 6: in addition to *neo* and *hyg* integration in *pim-1*, an unmodified *pim-1* copy is still present.

TABLE 1 Efficiency of homologous recombination

(a) Disruption of *pim-1* with *neo*

DNA	Number of cells E14 <i>pim</i> ⁺ / <i>pim</i> ⁺	G418 ^R	Homol. rec.
39	10 ⁸	1.5 × 10 ⁴	0/4
31	10 ⁸	8 × 10 ²	34/40 (85%)

(b) Disruption of *pim-1* with *hyg*

DNA	Number of cells E14 <i>pim</i> ⁺ / <i>pim</i> ⁺	Hyg ^R	Homol. rec.
pPGK <i>Hyg</i>	2 × 10 ⁷	700	
61 A ⁺	2 × 10 ⁷	235	N.D.
61 A ⁻	2 × 10 ⁷	115	N.D.
59 A ⁻	2 × 10 ⁷	13	6/11 (55%)
59 A ⁻	10 ⁸	48	20/31 (64%)

(c) Disruption of both *pim-1* alleles with *neo* and *hyg*

DNA	Number of cells E14 <i>pim</i> ⁺ / <i>neo</i> ⁺	Hyg ^R	Homol. rec.
59 A ⁻	10 ⁸	43	21/34 (62%)*

Efficiency of homologous recombination with *pim-1* targeting constructs. ES cell line E14 was grown on BRL conditioned medium as described¹⁰. Cells (10⁸ or 2 × 10⁷) were mixed with 150 or 100 µg of DNA, respectively, in a volume of 600 µl PBS buffer and electroporated using a Biorad Gene pulser (0.8 kV, 3 µF, electrode distance 0.4 cm). Cells were reseeded on 10-cm tissue culture dishes at a density of 10⁷ cells per plate. G418 selection (200 µg ml⁻¹) was started after one day and colonies were picked and expanded after 6 days; hygromycin B selection (110 µg ml⁻¹) was started after 2 days and colonies were picked after 12 days.

a. Electroporation of E14 ES cells with the *neo* constructs 39 (control) and 31 (targeting construct).

b. Electroporation of E14 ES cells with the *hyg* constructs 61 (control) plus and minus polyadenylation signal and 59A⁻ (targeting construct). A positive control for hygromycin B selection in ES cells was constructed as the Pim-Hyg fusion protein may have a reduced activity. This construct, pPGK-*hyg*, contains the PGK promoter¹¹, which is highly active in ES cells, an optimized translation initiation site¹², the coding sequence for the bacterial *hyg* gene, and the PGK polyadenylation signal. The number of ES cell colonies resistant to hygromycin B was markedly lower with 61A⁺ than with pPGK *hyg* (b). Only a twofold reduction in colony formation was seen if the *pim-1* polyadenylation signal was omitted from the *pim-hyg* fusion construct (compare 61A⁺ and 61A⁻). The absence of a promoter and a translational start codon further reduced the number of colonies resistant to hygromycin B tenfold (59A⁻). Fifty five per cent of these colonies (6 out of 11 tested) were the result of homologous recombination. In a separate experiment at a larger scale, 64% of the colonies resistant to hygromycin B (20 out of 31 tested) contained the *hyg* sequence integrated in one of the *pim-1* alleles. N.D., not determined.

c. Electroporation of one of the ES clones previously targeted with *pim-neo* construct 31 with the *pim-hyg* targeting construct 59A⁻.

* G418^SHyg^R: 6 → single knock-out. G418^RHyg^R: 15 → double knock-out.

differentiate *in vitro* on agarose-coated plates to which they cannot adhere. Characteristic embryoid bodies, containing contractile cells, developed in all cases. When reseeded on tissue culture plates, a variety of different cell types grew out from the embryoid body structures. No microscopically discernable differences were observed at this stage between cells with or without a functional *pim-1* gene. This suggests that in spite of its high expression in ES cells, *pim-1* is not required for normal ES cell proliferation and differentiation *in vitro*. Its function in ES cells is therefore unclear at present. As the Pim-1 serine/threonine protein kinase is thought to be involved in signal transduction, we might assume that differences in growth behaviour or differentiation potential might nevertheless become apparent by exposing the cells to different growth conditions and growth factors.

In conclusion, our results show that complete loss-of-function mutations in expressed genes can indeed be directly generated in diploid ES cells with high efficiency using targeting vectors

containing promoter/AUG-minus *neo* and *hyg* fusion genes. In our experiment the selection procedure used to inactivate the *pim-1* gene was particularly efficient. But data from other laboratories using different selection protocols indicate that a large variety of genes⁹, expressed and nonexpressed, can be targeted with a sufficiently high efficiency. Selection protocols employing a promoter-competent *neo* gene as a positive selection marker¹ should be readily modifiable for inactivation of the residual allele by substituting *hyg* or *neo*. In cases in which the gene of interest is expressed in cultured cells, there is now the option to study the effects of loss-of-function mutations directly *in vitro*, without the necessity to generate germ-line chimaeric mice as an intermediate step. Besides this application *in vitro*, chimaeric mice produced with ES cells lacking a particular gene function might reveal somatic abnormalities that are not accessible to analysis by crossing of heterozygous mutants because of early lethality. □

Received 14 August; accepted 10 October 1990.

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ACKNOWLEDGEMENTS. We thank C. Saris, J. Dornen and P. Laird for communicating unpublished results, and J. Dornen, P. Laird, C. Saris, M. van Lohuizen and M. van Roon for critically reading the manuscript. This work was supported by the Netherlands Cancer Foundation through a fellowship to H.T.R. and the Foundation for Medical and Health Research MEDIGON (E.R.M.).

A mutation in the tRNA^{Leu(UUR)} gene associated with the MELAS subgroup of mitochondrial encephalomyopathies

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MITOCHONDRIAL encephalomyopathies are usually divided into three distinct clinical subgroups: (1) mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes (MELAS); (2) myoclonus epilepsy associated with ragged-red fibres (MERRF); and (3) chronic progressive external ophthalmoplegia (CPEO) including Kearns–Sayre syndrome^{1–5}. Large deletions of human mitochondrial DNA and a transition mutation at the mitochondrial transfer RNA^{Lys} gene give rise to CPEO including Kearns–Sayre syndrome^{6–8} and MERRF^{9,10}, respectively. Here we report an A-to-G transition mutation at nucleotide pair 3,243 in the dihydrouridine loop of mitochondrial tRNA^{Leu(UUR)} that is specific to patients with MELAS. Because this mutation creates

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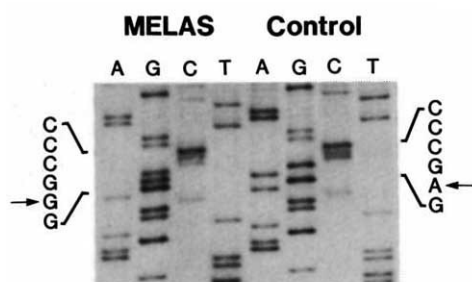


FIG. 1 Direct sequencing of the flanking region of nucleotide pair (np) 3,243 in a MELAS patient and a control. Nucleotide A in wild type is converted to a G at position 3,243. Because 22 tRNA genes are clustered in 11 locations, we synthesized 11 sets of 20-mer oligonucleotides with PCR-MATE (Applied Biosystems) as follows: np 520–539 (forward: f) and np 726–707 (reverse: r) for tRNA^{Phe}; np 1,549–1,568 (f) and np 1,722–1,703 (r) for tRNA^{Val}; np 3,130–3,149 (f) and np 3,423–3,404 (r) for tRNA^{Leu(UUR)}; np 4,101–4,120 (f) and np 4,531–4,512 (r) for tRNA^{Ile}, tRNA^{Gln} and tRNA^{Met}; np 5,470–5,489 (f) and np 5,928–5,909 (r) for tRNA^{Trp}, tRNA^{Ala}, tRNA^{Asn}, tRNA^{Cys} and tRNA^{Tyr}; np 7,375–7,394 (f) and np 7,914–7,895 (r) for tRNA^{Ser(UCN)} and tRNA^{Asp}; np 8,211–8,230 (f) and np 8,410–8,391 (r) for tRNA^{Lys}; np 9,912–9,931 (f) and np 10,555–10,536 (r) for tRNA^{Gly} and tRNA^{Arg}; np 11,798–11,817 (f) and np 12,519–12,500 (r) for tRNA^{His}, tRNA^{Ser(AGY)} and tRNA^{Leu(CUN)}; np 14,553–14,572 (f) and np 15,230–15,211 (r) for tRNA^{Glu}; and np 15,806–15,825 (f) and np 16,219–16,190 (r) for tRNA^{Thr} and tRNA^{Pro}. The double-stranded DNA fragments were amplified by the conventional PCR procedure using each set of primers²⁶. After electrophoretic separation, reamplification of an aliquot of elute from a selected gel slice with unidirectional or, if necessary, bidirectional asymmetric PCR was carried out to produce single-stranded templates²⁷. They were purified with a microconcentrator (Centricon 30, Amicon), and sequenced by the dideoxy chain termination method with Sequenase Version 2.0 (USB)²⁸.

an *ApaI* restriction site, we could perform a simple molecular diagnostic test for the disease. The mutation was present in 26 out of 31 independent MELAS patients and 1 out of 29 CPEO patients, but absent in the 5 MERRF and 50 controls tested. Southern blot analysis confirmed that the mutant DNA always coexists with the wild-type DNA (heteroplasmy).

Recent studies on the expression of defective mitochondrial (mt) DNA suggest that tRNA dysfunction is the cause of CPEO and MERRF^{9–13}. It is reasonable to assume that there are also tRNA abnormalities in MELAS, because all three mitochondrial encephalomyopathies are histologically similar and often have a defect(s) of respiratory-chain enzymes, in particular NADH coenzyme Q reductase and cytochrome *c* oxidase. There is no one-to-one correspondence between enzyme deficiencies and clinical entities, suggesting that mutations at tRNA or ribosomal RNA genes are more responsible for MELAS than are mutations of protein-coding regions. Therefore, we sequenced all mitochondrial tRNA genes of a MELAS patient from a pedigree in which the disease is maternally inherited. We directly sequen-

TABLE 1 Nucleotides A or G at position 3,243

	Total	A	G
MELAS	31	5	26
MERRF	5	5	0
CPEO	29	28	1
Control	50	50	0

ced the amplified DNA using the polymerase chain reaction (PCR) with 11 sets of 20-residue oligonucleotide primers (Fig. 1). Compared with reference sequences¹⁴, only one nucleotide substitution was detected, occurring at nucleotide pair 3,243 in the coding region of the tRNA^{Leu(UUR)} gene. This corresponds to the first base of the dihydrouridine loop and is strictly invariant from humans to sea urchins^{14–23} (Fig. 2). This base also forms a tertiary hydrogen bond with the T residue of the amino-acid acceptor arm positioned around a turn of L-shape structure²⁴. Hence, any substitution at this position can greatly affect tRNA^{Leu(UUR)} function.

This mutation allowed us to develop a simple molecular diagnostic test for the disease which uses the restriction endonuclease *ApaI*. This enzyme can cleave the mutant sequence (GGGCCC) but not the wild-type sequence (GAGCCC). A 294-base-pair (bp) fragment encompassing the mutation site was therefore amplified by PCR and digested with *ApaI*. Each set of PCR fragments from 26 of 31 independent MELAS patients was cleaved to give three fragments (the original 294-bp fragment and the new 182-bp and 112-bp fragments), but those from 50 normal controls remained as copies of the 294-bp fragment even after overnight digestion. Thus the mutation at nucleotide pair 3,243 seems to be most responsible for MELAS. Two other members in the pedigree, including the original patient, have the mutation. Five patients without the mutation, however, show all the clinical and histological features of MELAS, indicating that there is another molecular cause of MELAS. An examination of the digestion profile always shows the coexistence of mutant and wild-type genomes (heteroplasmy). This was confirmed by Southern blotting: populations of mutant DNA ranged from 50 to 92% using densitometry (Fig. 3).

To determine whether this mutation is specific to MELAS, we examined samples from other mitochondrial diseases (Table 1). All of the five MERRF patients examined had a point mutation at nucleotide pair 8,344 but lacked the mutation at nucleotide pair 3,243. Out of 29 CPEO patients examined, 28 lacked the mutation. Of these 28, 25 had single large deletions, two had multiple deletions and one had no deletions, as observed on Southern blot analyses. The CPEO patient who carried the mutation had no large deletion of mtDNA. His mother and sister also showed clinical features of mitochondrial diseases, having CPEO and muscle weakness; the sister had periodic central nervous system symptoms with focal low lucency (shown

FIG. 2 Interspecies similarity of the mitochondrial tRNA^{Leu(UUR)} genes. An adenine, or the first base of the dihydrouridine (DHU) loop in tRNA^{Leu(UUR)}, is boxed and is evolutionarily conserved among human¹⁴, bovine¹⁵, mouse¹⁶, rat¹⁷, chicken¹⁸, frog¹⁹, two species of fly^{20,21} and two species of sea urchin^{22,23}. The base forms a hydrogen bond to T(*), (U in the tRNA) to construct a functional tertiary L-shape structure, so the substitution probably causes the dysfunction leading to suppression of protein synthesis. A.A., amino acid; A.C., anticodon; V., variable; T., TψC loop; D. YAKUBA, *Drosophila yakuba*; D. MEL., *Drosophila melanogaster*; and S.U., sea urchin (PL, *Paracentrotus lividus*; SP, *Strongylocentrotus purpuratus*).

	A.A. stem	DHU stem	DHU loop	DHU stem	A.C. stem	A.C. loop	A.C. stem	V. stem	T. loop	T. stem	T. loop	A.A. stem
HUMAN	GTAAAGTGGCAG	A	GCCCGGTAATGCGATAAACTTAAACCTTTACAGTCAGAGTTCAATTCCTCTCTTAACA	A	GCCCGGTAATGCGATAAACTTAAACCTTTATATCCAGAGTTCAATTCCTCTCTTAACA	A	GCCAGGAAATGCGTAAGACTTAAACCTTTGTTCCAGAGTTCAATTCCTCTCTTAACA	A	GCCAGGAAATGCGTAAGACTTAAACCTTTGTTCCAGAGTTCAATTCCTCTCTTAACA	A	GCCAGGAAATGCGTAAGACTTAAACCTTTGTTCCAGAGTTCAATTCCTCTCTTAACA	A
BOVINE	GTAAAGTGGCAG	A	GCCCGGTAATGCGATAAACTTAAACCTTTATATCCAGAGTTCAATTCCTCTCTTAACA	A	GCCCGGTAATGCGATAAACTTAAACCTTTATATCCAGAGTTCAATTCCTCTCTTAACA	A	GCCAGGAAATGCGTAAGACTTAAACCTTTGTTCCAGAGTTCAATTCCTCTCTTAACA	A	GCCAGGAAATGCGTAAGACTTAAACCTTTGTTCCAGAGTTCAATTCCTCTCTTAACA	A	GCCAGGAAATGCGTAAGACTTAAACCTTTGTTCCAGAGTTCAATTCCTCTCTTAACA	A
MOUSE	ATTAGGGTGGCAG	A	GCCAGGAAATGCGTAAGACTTAAACCTTTGTTCCAGAGTTCAATTCCTCTCTTAACA	A	GCCAGGAAATGCGTAAGACTTAAACCTTTGTTCCAGAGTTCAATTCCTCTCTTAACA	A	GCCAGGAAATGCGTAAGACTTAAACCTTTGTTCCAGAGTTCAATTCCTCTCTTAACA	A	GCCAGGAAATGCGTAAGACTTAAACCTTTGTTCCAGAGTTCAATTCCTCTCTTAACA	A	GCCAGGAAATGCGTAAGACTTAAACCTTTGTTCCAGAGTTCAATTCCTCTCTTAACA	A
RAT	ATTAGGGTGGCAG	A	GCCAGGAAATGCGTAAGACTTAAACCTTTGTTCCAGAGTTCAATTCCTCTCTTAACA	A	GCCAGGAAATGCGTAAGACTTAAACCTTTGTTCCAGAGTTCAATTCCTCTCTTAACA	A	GCCAGGAAATGCGTAAGACTTAAACCTTTGTTCCAGAGTTCAATTCCTCTCTTAACA	A	GCCAGGAAATGCGTAAGACTTAAACCTTTGTTCCAGAGTTCAATTCCTCTCTTAACA	A	GCCAGGAAATGCGTAAGACTTAAACCTTTGTTCCAGAGTTCAATTCCTCTCTTAACA	A
CHICKEN	GCTAGCGTGGCAG	A	GCTCGGCAATGCGAAAGGCTTAAGCCCTTTAT-CCAGAGTTCAATTCCTCTCTTAACA	A	GCTCGGCAATGCGAAAGGCTTAAGCCCTTTAT-CCAGAGTTCAATTCCTCTCTTAACA	A	GCTCGGCAATGCGAAAGGCTTAAGCCCTTTAT-CCAGAGTTCAATTCCTCTCTTAACA	A	GCTCGGCAATGCGAAAGGCTTAAGCCCTTTAT-CCAGAGTTCAATTCCTCTCTTAACA	A	GCTCGGCAATGCGAAAGGCTTAAGCCCTTTAT-CCAGAGTTCAATTCCTCTCTTAACA	A
FROG	GCTAGCGTGGCAG	A	GCTCGGCAATGCGAAAGGCTTAAGCCCTTTAT-CCAGAGTTCAATTCCTCTCTTAACA	A	GCTCGGCAATGCGAAAGGCTTAAGCCCTTTAT-CCAGAGTTCAATTCCTCTCTTAACA	A	GCTCGGCAATGCGAAAGGCTTAAGCCCTTTAT-CCAGAGTTCAATTCCTCTCTTAACA	A	GCTCGGCAATGCGAAAGGCTTAAGCCCTTTAT-CCAGAGTTCAATTCCTCTCTTAACA	A	GCTCGGCAATGCGAAAGGCTTAAGCCCTTTAT-CCAGAGTTCAATTCCTCTCTTAACA	A
D. YAKUBA	TCTAATATGGCAG	A	T-----TAGTCCAATGGATTTAAGCTCCATAT-ATAAGTAT--TTTACTTTTATTAGAA	A	T-----TAGTCCAATGGATTTAAGCTCCATAT-ATAAGTAT--TTTACTTTTATTAGAA	A	T-----TAGTCCAATGGATTTAAGCTCCATAT-ATAAGTAT--TTTACTTTTATTAGAA	A	T-----TAGTCCAATGGATTTAAGCTCCATAT-ATAAGTAT--TTTACTTTTATTAGAA	A	T-----TAGTCCAATGGATTTAAGCTCCATAT-ATAAGTAT--TTTACTTTTATTAGAA	A
D. MEL.	TCTAATATGGCAG	A	T-----TAGTCCAATGGATTTAAGCTCCATAT-ATAAGTAT--TTTACTTTTATTAGAA	A	T-----TAGTCCAATGGATTTAAGCTCCATAT-ATAAGTAT--TTTACTTTTATTAGAA	A	T-----TAGTCCAATGGATTTAAGCTCCATAT-ATAAGTAT--TTTACTTTTATTAGAA	A	T-----TAGTCCAATGGATTTAAGCTCCATAT-ATAAGTAT--TTTACTTTTATTAGAA	A	T-----TAGTCCAATGGATTTAAGCTCCATAT-ATAAGTAT--TTTACTTTTATTAGAA	A
S.U. (PL)	GCTAAATAGCAA	A	G--TGGTTAATGCGAAGGCTTAAGCCCTTTAT-CCAGAGTTCAATTCCTCTCTTAAGCT	A	G--TGGTTAATGCGAAGGCTTAAGCCCTTTAT-CCAGAGTTCAATTCCTCTCTTAAGCT	A	G--TGGTTAATGCGAAGGCTTAAGCCCTTTAT-CCAGAGTTCAATTCCTCTCTTAAGCT	A	G--TGGTTAATGCGAAGGCTTAAGCCCTTTAT-CCAGAGTTCAATTCCTCTCTTAAGCT	A	G--TGGTTAATGCGAAGGCTTAAGCCCTTTAT-CCAGAGTTCAATTCCTCTCTTAAGCT	A
S.U. (SP)	ACTAAGTAGCAA	A	G--TGGTTAATGCGAAGGCTTAAGCCCTTTAT-CCAGAGTTCAATTCCTCTCTTAAGCT	A	G--TGGTTAATGCGAAGGCTTAAGCCCTTTAT-CCAGAGTTCAATTCCTCTCTTAAGCT	A	G--TGGTTAATGCGAAGGCTTAAGCCCTTTAT-CCAGAGTTCAATTCCTCTCTTAAGCT	A	G--TGGTTAATGCGAAGGCTTAAGCCCTTTAT-CCAGAGTTCAATTCCTCTCTTAAGCT	A	G--TGGTTAATGCGAAGGCTTAAGCCCTTTAT-CCAGAGTTCAATTCCTCTCTTAAGCT	A

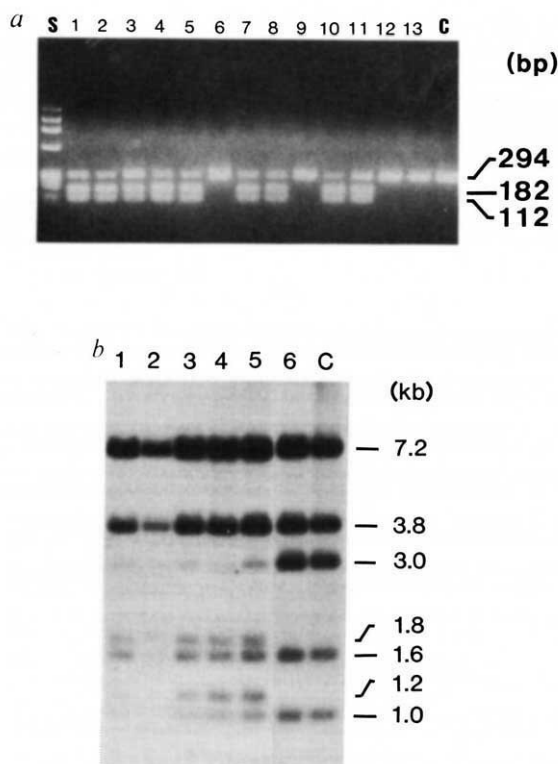


FIG. 3 The digestion profiles of PCR fragments amplified with a set of primers for tRNA^{Leu(UUR)} and Southern blot analyses. *a*, Point mutation (A to G) at nucleotide pair 3,243 was found in MELAS patients but not in controls. Size-standard DNA (S) was Φ X174 DNA digested by *Hae*III. Fragments of 294 bp amplified by conventional PCR using a set of primers for tRNA^{Leu(UUR)} (Fig. 1) were digested with the restriction endonuclease *Ap*I for more than 6 h. With samples from muscles of MELAS patients (lanes 1–5, 7, 8, 10, 11), an original and two new fragments (182 bp and 112 bp) could be recognized on electrophoresis after staining with ethidium bromide. By contrast, fragments of 294 bp from some MELAS patients (lanes, 6, 9, 12, 13) and that amplified with the placental DNA of a control as template (lane C) remained unchanged after the digestion with *Ap*I. *b*, Southern blot analyses of total DNAs from patients and a control after *Ap*I digestion. In most MELAS patients (lanes 1–5), varying amounts of 3.0-kilobase (kb) fragments were cleaved into two smaller ones of 1.8 and 1.2 kb because of the site-gain mutation at nucleotide pair 3,243, and the rest of the 3.0-kb fragments remained unchanged, indicating the presence of wild-type DNA (heteroplasmy). One MELAS patient (lane 6) and one control (lane C) show no 1.8- and 1.2-kb bands after *Ap*I digestion.

by tomography) similar to that of MELAS²⁵. Although we could not evaluate the importance of the site-gain mutation in the male CPEO patient, it is likely that the mutant mtDNA is not abundant enough for the clinical symptoms to be manifest. Alternatively, the effect of the abnormalities leading to CPEO might be much more severe than that of the point mutation leading to MELAS. We conclude that the mutation at nucleotide pair 3,243 is specific, although not exclusive, to MELAS.

How abnormalities of tRNAs cause functional and morphological changes of mitochondria, and why types of tRNA mutations are generally correlated with types of mitochondrial diseases, remain to be answered. Other factors, particularly nuclear factors, could be involved in the pathogenesis of mitochondrial diseases. Nonetheless, the mutation at nucleotide pair 3,243 seems to be the main cause of MELAS, and all three distinct subgroups of mitochondrial diseases may share similar mitochondrial tRNA abnormalities. □

Received 28 August; accepted 12 October 1990.

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ACKNOWLEDGEMENTS. We thank N. Takahata for critically reading the manuscript. This work was supported in part by the Ministry of Health and Welfare and by the Scientific Research from the Japanese Ministry of Education, Science and Culture (I.N. and S.H.).

Evolution of a polymeric globin in the brine shrimp *Artemia*

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SEVERAL invertebrate species possess haemoglobins in which each polypeptide contains multiple haem-binding domains¹, possibly reflecting the fusion of multiple monomeric globin transcriptional units at the gene level. We have now analysed the transcript of such a polymeric globin gene from the brine shrimp *Artemia*, which expresses three polymeric haemoglobins, each of relative molecular mass 260,000 (M_r 260K). These are formed by the variable association of two different subunit types, α and β (refs 2, 3). Haemoglobins I and III are homodimers of α and β subunit types, respectively, and haemoglobin II is a heterodimer ($\alpha\beta$). The individual globin chains are of similar size (M_r 130K), but the exact nature of the differences between the two subunit types is unclear. Analysis of complementary DNA clones encoding one of the subunits of the *Artemia* dimeric haemoglobin showed that the globin messenger RNA encodes nine myoglobin-like domains, connected by linking peptides. The residues in the linkers are characteristic of those found generally in such protein linkers, and include turn-promoting amino acids. Each domain also contains the conserved residues that are required for functional haem-binding, and from analysis of the sequences it was predicted that they all can adopt the classic myoglobin-like fold. Analysis of the derived amino-acid sequences indicated that the individual domains are duplicated monomers that fused to form the polymeric globin some 200 Myr ago. The fusion of multiple transcriptional units for the evolution of a polymeric globin gene may have been a general mechanism for the appearance of such polymeric haemoglobins in invertebrates.

We isolated several *Artemia* globin complementary DNA clones⁴ and determined the complete DNA and derived amino-acid sequence of the largest (Fig. 1). The authenticity of the

Analysis of the deduced amino-acid sequence revealed the presence of nine separate haem-binding domains, named T1 to

is marked with an arrow. Differences between the translated cDNA sequence and the amino-acid sequence of isolated peptides are shown below the sequences as asterisks. The termination signal (4,216–4,219) is also marked by an asterisk. Potential polyadenylation signals (beginning at 4,280 and 4,294, respectively) are underlined.

T9 (Fig. 1), each containing residues characteristic of the helices A to H of monomeric globins. From the cDNA sequence we predicted a termination codon at position 4,216 in the nucleotide sequence. The globin polypeptide would, therefore, end 16 residues beyond the H23 of a myoglobin. The 3' untranslated region of 96 nucleotides is similar in size to that seen in virtually all monomeric globin transcripts⁸, including those of the dipteran midge *Chironomus*, the only other arthropod for which globin gene sequence is available⁹.

Each of the haem-binding domains displayed weak homology to other globins, with no more than 27% sequence similarity with any other globin. Even though the amino-acid sequence homology between *Artemia* and other globins was relatively low, structural similarity to myoglobin was high. Alignment of

the individual haem-binding domains around their conserved residues and around those residues conserved in other globins¹¹, such as those involved in haem contacts or in determining the relationship of the helical segments to each other, suggested that the *Artemia* domains possessed the classical globin helices A to H with their corresponding turns (Fig. 2). Differences in the length of helices could mostly be accommodated in turns, as would be expected in variants on a conserved tertiary structure. All domains contained the invariant CD1 Phe and F8 His, and the highly conserved E7 His (ref. 11; Fig. 2, asterisks). Other conserved features of the globin pattern included Trp at A12, Pro at C2 and Val at E11. Several additional residues were highly conserved between the *Artemia* domains, including Phe at B10, Tyr at C4, Gly at F5 and Phe at G5. Each of the domains

	Post H/linker	A	8	12	15	B	6	10	C2	4	CD	4	7	D	E	5	7	8	11
				*			*		*		*					*	*		
Myoglobin		VL	SEGE	MQVLVH	MAKVE	A	DVAGH	GGQDILIR	LRFKS	HPETLEK	FDR	FKHLK	TEAEMKA	SEDLKKHGVITVLTALGAILK					
Chironomus		L	SADQISTV	QASFDKVK	G		DPVGILYAVFKA	DPSIMAK	FTQ	FAGKD	LESIGK	TAPFETHANRIVGFFSKIIG							
Artemia 1			DKATIKRTWATVT				DLPSFGNRNVFLSVFAA	KPEYKNL	FVE	FRNIP	ASELAS	SERLLYHGGRVLSSIDEAIA							
Artemia 2	LRRQIDLEVT	GL	SCVDVANIQESWSKVS	G			DLKTTGSSVWFQRMING	HPEYQQL	FRQ	FRDVD	LDKLGE	SNSFVAHVFRVVAAFDGIH							
Artemia 3	SSLKRVDPIT	GL	SGLEKNAILSTMGKVR	G			NLQEVGKATFGKLFKA	HPEYQQM	FRF	SQGMF	LASLVE	SPKFAAHTQRVVSALDQTL							
Artemia 4	RQADIVDPVT	HL	TGRQKEMIKASMSKAR	T			DLRSLGQELFMRMFKA	HPEYQTL	FVNKGADVP	LVSLRE	DERFISHMANVLGGFDITLLQ								
Artemia 5	ATSEADPVT	GL	YGKEIVALRQAFAAVT	P			RNVEIGKRVFAKLFKA	HPEYKNL	FKK	FEQYS	VEELPS	TDAFYHISLVMMRPFSSIGK							
Artemia 6	FQLGQVDSNT	L	TALEKQSIQDINSNLR	S			TGLQDLAVKIFTRLFSA	HPEYKLL	FTGR	FGN	VDNINE	NAPFKALHRLVLSAFDIVIS							
Artemia 7	LQLERINPIT	GL	SAREVAVVKQTWNVLK	P			DLMGVGMRIFKSLFEA	FPAYQAV	FPK	FSDVP	LDKLED	TPAVGKHSISVTTKLDELIO							
Artemia 8	QGSYKQDPVT	GI	TDAEKALVQESWDLK	P			DLGLGRKIFTRKVFKA	HPDYQIL	FTRTGFGDTP	LTKLDO	NPAFGTHIIRKVRADFHVIO								
Artemia 9	IGLKEVNPQN	AF	SAYDIQAVQRTWALAK	P			DLMGKGAMVFKQLFTD	HG	YQPL	FSN	LAQYE	ITGLEG	SPELNTHARNVMAQLDITLVG						
	EF	F	4	8	FG	G		16	GH	5	H		10		22			Post H/linker	
			*	*		*													
Myoglobin	KGHHEAE	LKPLAQSHAT	KHKI	PIKYLEFISEAIIHVLHSR	HPGDF	GADAQAGAMNKALELFRKDIAAKY	KELGYQG												
Chironomus	ELPN	IEAD	VNTFVASHKP	RGV	THDQLNFRAGFVSVMKAH	T D F	A	GAEAAAGATLDTFFGHIFSKM											
Artemia 1	GIATPDRAVKT	LLALGERHIS	RGT	VRRHFEAFSYAFIDELKQR	G	V	ESADLAAMRRGNDINVNLEAGL	LRRQIDLEVT	GL										
Artemia 2	ELDNQFIVST	LKKLGEQHIA	RGT	DISHFQNRVTLLEYLKEN	G	M	NGAQKASWNKAFDAFEKYISMGL	SSLKRVDPIT	GL										
Artemia 3	ALNRPSDFVYM	IKELGLDHIN	RGT	DRSHFENYQVVFIEYLKET	LGDSL	DEFTVKSFNHVFVVISFLNEGL	RQADIVDPVT	HL											
Artemia 4	NLDESSYFIYS	LRNLGDAHIQ	RKA	GTQHSRFEAILIPILQES	Q	G L	DAASVEAMKGFVDSIGVIAQGLKVATSEADPVT	GL											
Artemia 5	VIDDNVSFVYL	LKKLGREHIK	RGL	SRKQFDQFVELYIAEISSE	L	S	DT GRNGLEKVLTFATGVIEQGL	FQLGQVDSNT	L										
Artemia 6	TLDSEHLIRQ	LKDLGLEHTR	LGM	TRSHFDNFATAFLSVAQDI	APNQL	TVLGRESLNKGFKLHGVIEEGL	LQLERINPIT	GL											
Artemia 7	TLDEPANLALL	ARQLGEDHIV	LRV	NKPMKFSFGKVLVRLLEND	LQGRF	SSFASRSWHKAYDVIVEYIEEGL	QGSYKQDPVT	GI											
Artemia 8	ILGKPKTLMAY	LRVSGADHIA	TNV	ERRHFQAFSNALIPVMQHD	LKAQL	RPDAVAAMRRGLDRIIGIIDQGL	IGLKEVNPQN	AF											
Artemia 9	SLGNSIELQGS	LAQLGKDHPV	RKV	NRVHFKDFAEHFIPLMKAD	LGDEF	TPLAESAMKRAFDVMIATIEQQG	EGSSHALSSF	LT	NPVA*										

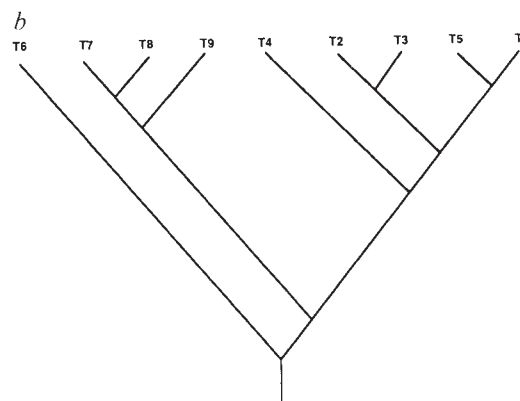
FIG. 2 Alignment of the derived amino-acid sequences (single-letter amino-acid code) for the nine domains of *Artemia* haemoglobin against sperm-whale myoglobin and globin 3 of the dipteran midge *Chironomus*. The translated cDNA sequence was subdivided into nine successive globin-like domains (T1–T9) and aligned over their conserved sites (asterisks) using the homology editor HOMED¹⁰. This alignment was then refined by the addition of both sperm-whale myoglobin and *Chironomus* globin 3 sequences.

Differences in lengths were able to be accommodated in turns. Final adjustment of the alignment was performed with the aid of template II of Bashford *et al.*¹⁰, with an associated database of 226 globin sequences. Alignments of each of the helices were adjusted to minimize penalty scores against the Bashford template. The post H/linker region of each domain is duplicated in the alignment to allow comparison to both the post H and pre A regions of the other globins.

	Number of amino differences									Total residues
	T1	T2	T3	T4	T5	T6	T7	T8	T9	
T1		101	109	100	104	113	108	110	117	150
T2	32.7		96	108	114	108	110	108	125	154
T3	27.3	37.9		108	115	107	108	117	121	157
T4	33.3	29.9	31.2		124	110	114	106	119	159
T5	30.7	26.0	26.8	22.0		115	120	123	125	152
T6	24.7	29.9	31.9	30.8	24.3		123	108	114	157
T7	28.0	28.6	31.2	28.3	21.1	21.7		100	108	159
T8	26.7	29.9	25.5	33.3	19.1	31.2	30.0		110	160
T9	22.0	18.8	22.9	25.2	17.7	27.4	31.2	30.8		

FIG. 3 *a*, Comparison of the deduced domain sequences of the α -globin polypeptide. The nine separate haem-binding domains were maximally aligned on the basis of sperm-whale myoglobin and *Chironomus* globin 3 amino-acid sequences (see Fig. 2). Each domain was defined as beginning at residues of the recognized A helix and ending at the last residue of the linker region. Differences in amino-acid sequences at each position of the domain sequence were calculated. Insertions or deletions of amino acids were scored as differences between the two sequences. Results are expressed as number of amino-acid differences and percentage of amino-acid identity. Total residues refer to the number of amino acids contained in each haem-binding domain. *b*, Evolutionary tree generated using the tree-building programs included in the phylogeny inference package PHYLIP¹⁷ and each of the cDNA-derived haem-binding domains (T1–T9) as separate entries.

% amino acid identity



was tested for its conformity to the consensus globin template II of Bashford *et al.*¹⁰. The total of penalties per domain ranged from 3.4 to 8.3 (mean 5.1 per domain, or 0.85 per pattern) and there were no violations of 'required' (class 2) side chains. All those residues at buried sites in the Bashford template II are either hydrophobic or neutral in *Artemia*, with the exception of residues at G15 in both T1 and T5 (Glu) and at E14 in T5 (Arg), T7 (Lys) and T9 (Gln). The effect that these deviations have on the globin fold is unclear, although they are confined to only two specific sites. From this analysis we predict that each of the domains can adopt the structure of the classic myoglobin fold. This conclusion is supported by template model building of the E1 domain⁶ (analogous to T3), which demonstrated that this domain is homologous in primary and probably also in tertiary structure with the typical globin polypeptides of low M_r . From this analysis we predict that all nine domains are functional.

Each domain is connected by a short linker peptide (Fig. 1, boxes). The linkers are of similar length and composition, containing Thr, Ser, Asp and especially Gly and Gln, all associated with natural protein linkers¹². The highly conserved nature of the linkers between the *Artemia* domains suggests that they adopt similar structures. Although it is unclear whether the linkers have evolved from sequences analogous to the post-H or pre-A region of other globins, a model could exist in globin V of the lamprey *Petromyzon*, of which the crystal structure is known¹³. Preceding the A helix of *Petromyzon* is a sequence of 11 residues of remarkably similar character to those in the

Artemia linker sequences. There are five steric or character similarities between the H region and the following A, with two turn-provoking residues (Gly and Pro) in similar positions in both sequences. If the *Artemia* sequence does conform to a similar template, then the major part of the linker sequence would be applied to the surface of the molecule with the inter-domain bridge confined to the proximal part.

The *Artemia* polymeric globin is assumed to have evolved through a process of duplication and fusion of a monomeric globin gene ancestral to the invertebrate lineage¹⁵ and a short linker structure is assumed to have evolved to facilitate the fusion of multiple haem-binding domains. From estimates of the number of amino-acid differences between the maximally aligned domains it can be estimated that these duplications occurred at least 200–250 Myr ago¹⁴. The domains, although displaying high structural similarity, have diverged significantly at the amino-acid level (Fig. 3*a*). Phylogenetic tree building suggests a series of duplication events that may have led to the generation of the polymeric globin (Fig. 3*b*). The tree is consistent with the observed domain homologies. Whereas gene duplication is a well established process by which the globin gene family has arisen in many divergent species¹¹, the fusion of separate globin transcriptional units has not been observed before. It is likely that other polymeric globins also evolved by a process similar to that of *Artemia* globin, suggesting that duplication and fusion of structural units represent a general mechanism for globin gene evolution in invertebrates. □

Received 23 April; accepted 4 October 1990.

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ACKNOWLEDGEMENTS. This work was supported in part by the Medical Research Council of New Zealand (A.M.M.).

A multiplex DNA sequencing system coupled with chemiluminescent detection chemistry is available in kit form for high-throughput data acquisition.

a

alkaline phosphatase

AMPPD

AMP'D

LIGHT

b

LIGHT

MEMBRANE

Dioxetane

Biotinylated alkaline phosphatase

Streptavidin

Biotinylated Plex probe

Oligomer

Crosslinking

This throughput makes it possible to carry out a 20- to 30-kb random shotgun project, with five- to six-fold coverage, in less than a month. It should be noted that, to obtain sequence information for closure of the project, the multiplexed DNA pools can be used with custom biotinylated primers to sequence internal regions of any clone in that pool.

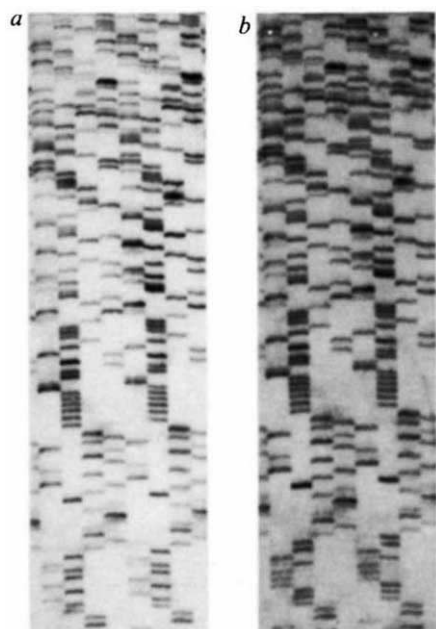


FIG. 2 Chemiluminescing band pattern of membrane recorded on X-ray film, 10-minute exposure. *a*, First probing and *b*, eighth probing.

The classical method of multiplex sequencing is being used in the large-scale sequencing of the *Escherichia coli* genome (G.M. Church, personal communication). The chemiluminescent multiplex system can easily be scaled up to obtain an order of magnitude increase in throughput for genome projects of this size. As with the classical method, vector pools could be processed in a microtitre plate format (96 samples), the samples run on eight gels in one day, and the hybridization/chemiluminescent detection performed in batch mode, processing up to 80 membranes at a time. In our laboratory, the chemiluminescent multiplex approach will be used to obtain random start points for a genomic walking strategy, by which we will sequence the entire *Mycoplasma capricolum* genome.

Benefits and future uses

The ease of use and speed of the new detection chemistry compares well with traditional radioisotopic methods and only a modest investment in new equipment is required to implement the technology. The electroblotting apparatus can be made in house or purchased commercially and is well worth the investment when one considers that the time required to electroblot the gel is less than that required to dry a gel for the standard radioisotopic method.

The long half-life of AMP⁺ D permits the use of other detection formats such as polaroid film and CCD cameras⁹. There is no significant band broadening caused by the diffusion of the anion and it has been suggested that this is due to localization of the anion through hydrophobic interactions with the nylon membrane. The

Equipping the lab

Year-end products include instrumentation for the automatic injection of oocytes with DNA, a twin-block thermal cycler where each block can be controlled independently, and bibliographic software that brings order to chaos.

THE 1990/1992 53-page **colour catalogue of laboratory consumables** is now available from Elkay (*Reader Service No. 101*). The Elkay/Labsystems product line introduces 45 new product categories, including 5- and 10-ml transport tubes, printed 15- and 50-ml centrifuge tubes, custom-printed tubes, serological pipettes, biohazard bags, and Labsystems' Finnpiptettes, Microstrips and Combiplates. A section that is dedicated to chemical resistance includes information on the physical properties of plastics.

For the **automatic DNA injection of oocytes**, Drummond Scientific recommends its remote-controlled nanolitre pipette called the Auto/Oocyte Injector (*Reader Service No. 102*). The Auto/Oocyte Injector, which can be attached to most micropositioners, features a remote-controlled, non-rotating plunger that is designed to eliminate tip vibration. The Injector is microprocessor-controlled with a preprogrammed fixed volume of 46 nanolitres per injection. The total capacity is 5 µl, which permits up to 100 sequential injections, says Drummond.

Bio-Products has developed a novel range of **surface-active plates**, called Acti-Plates, which can be used in immunoassay development, nucleic acid probe screening and cell panning (*Reader Service No. 103*). Acti-Plate A has surface aldehyde groups for the covalent immobilization

of a wide range of ligands, including small or hydrophilic ligands that adsorb poorly — or not at all — to conventional plates. The solid phase has been engineered to provide low non-specific binding characteristics after the coupling procedure, says the company. Acti-Plates are also available with biotin, strept-avidin or Protein A pre-coupled to the solid phase.

At the molecular level

Ilume-Plume is a handy **marker** that is designed for the permanent marking of autoradiograms without the use of radioactivity (*Reader Service No. 104*). The autoradiogram marker, which contains a luminescent pigment, can be used to mark orientations on autoradiograms of Southern, western and northern blots, as well as for the identification of DNA sequencing gels. The \$3.50 (US) Ilume-Plume marker is available in a 30-ml bottle that comes with a plastic tip.

Laser Laboratory Systems is distributing the Ericomp **TwinBlock thermal cycler**, which features independent microprocessor control of each block (*Reader Service No. 105*). The £3,800 (UK) thermal cycler can be used to incubate up to 58 samples simultaneously or, alternatively, to perform two independent experiments simultaneously, each with up to 29 samples. According to Laser, the TwinBlock system accommodates 0.6- to 1.5-ml microcentrifuge tubes and pro-

high quantum yield of the reaction, allowing short exposure times, and the stability of reagents further increases the usefulness of the chemiluminescent detection chemistry.

Several steps in the process readily lend themselves to automation. The implementation of direct blotting electrophoresis¹⁰ would improve the ease and reliability of DNA transfer to the nylon membrane and allow longer sequences to be read from each reaction set. Automation of the hybridization and detection protocol in probing chambers¹¹ would further increase the quality of the data and the speed of data acquisition. In the near future, it is likely that the use of PCR amplification to process the DNA pools and of automated film readers in conjunction with the above instrumentation will completely automate what was once a laborious and time-consuming process,

opening the door to new horizons in molecular biology. □

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Laser's TwinBlock thermal cycler.

vides temperature control over the range of 25 °C to 110 °C, or 4 °C to 110 °C if a recirculating cooler is used. Well-to-well temperature uniformity is $\pm 1\%$ during 'holds' of one second to 18 hours. The thermal cycler features a built-in temperature probe for continuous monitoring of well temperatures, memory facilities for the storage of run programmes, and interface ports for the attachment of a printer or chart recorder if hard-copy output is required.

Flowgen has launched a new line of **electrophoresis chambers** that are designed to improve and simplify DNA and protein separations (*Reader Service No. 106*). Vertical sequencing rigs are available that can accommodate either 40 × 30-cm or 50 × 20-cm gels. Both feature a fully adjustable heat dissipation plate, which helps produce 'smile-free' runs and eliminate edge effects, says Flowgen. Other design features include easy-lift covers, shrouded power leads for safe operation, and buffer drain taps. Two horizontal gel chambers are also available where gels are cast in removable UV-transparent trays. Recirculation ports allow buffer cooling, if required.

Applied Biosystems has developed an **automated fluorescent fragment analyser** called Gene Scanner (*Reader Service No. 107*). The system is based on ABI's 'four-dye, one-lane' gel scheme. The instrument offers quantitative DNA fragment sizing and a hundred-fold increase in sensitivity over conventional radioactive and Southern blot methods, says ABI. The four different fluorescent dyes are used for the covalent labelling of individual samples, although ethidium bromide can be used instead. Up to four samples, or three samples and an internal standard, can then be run in a single gel lane. After the samples are loaded into the system's horizontal gel, Gene Scanner will perform all procedures from real-time fragment detection, during electrophoresis, to the presentation of data. Results can be displayed in a variety of formats: as a four-colour digitized gel, as individual sample electrophoretograms, and as a table showing fragment size, dye

labelling and fluorescent peak area and height.

Handy helpers

Tecnomara has introduced an extendable, small-scale **roller bottle system** called Cellroll, which can turn up to eight roller bottles at speeds of between 0.1 and 2.5 r.p.m. (*Reader Service No. 108*). The control unit can be dismantled from the basic unit for operation in a CO₂ incubator.

Manostat's **pen-style colony counter** is designed to reduce the likelihood of error when counting microbiological colonies (*Reader Service No. 109*). The counter

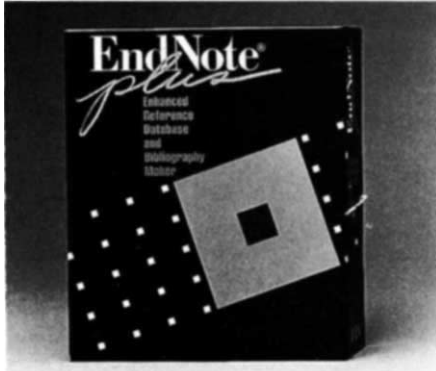


Counting colonies Manostat's way.

beeps, counts and marks all in one motion. The cumulative count is displayed on a four-digit LCD. The counter features an on/off switch to preserve the 3- $\frac{1}{2}$ battery that powers the unit.

Scientific software

Niles and Associates is now shipping Version 1.3 of EndNote Plus, **bibliography software** for the Macintosh computer (*Reader Service No. 110*). The \$249 (US) EndNote Plus package, which has more powerful search capabilities than an earlier EndNote package, allows the user to conduct fast one-step boolean searches, says Niles. In addition to all of EndNote's functions, the new software includes a QuickFind feature — a full text index — that is designed to allow the user to find records almost instantly, even in a file with thousands of references. EndNote Plus also allows the user to sort a database



EndNote Plus software makes light work of bibliographic chores.

on up to five fields, and set each to ascending or descending order. This can, says Niles, facilitate the process of building a bibliography sorted by subject. Other features include a 'Find duplicates' option that displays entries with identical author, year and title fields, and a glossary of journal abbreviations. EndNote Plus requires a Macintosh 5.12KE or higher.

SlideWrite Plus 4.0 from Advanced Graphics Software enables users to turn scientific and technical raw data into publication-quality charts, graphs and slides (*Reader Service No. 111*). The **graphics software** package is supplied with four fonts — Sans, Sans Bold (like Helvetica), Roman, and Roman Bold (like Times Roman) — and hard-to-find characters such as bullets, mathematics symbols, copyright and trademark symbols, and a full Greek upper- and lower-case alphabet. The new graphical users interface comes complete with pull-down menus, pop-up dialogue boxes and context-sensitive help. Advanced users can choose from using a mouse, mnemonic keyboard commands, menus and dialogue boxes. The \$445 (US) SlideWrite program runs on DOS machines.

Lab safety

Mop up those hazardous spills of acids and other chemicals with Spill-X kits from EM Science (*Reader Service No. 112*). Five **safety kits** are available, all containing a set of non-toxic agents that are designed to neutralize, solidify or, otherwise, stabilize spills involving acids, caustic solutions, formaldehyde, and the vapours that they emit. The Spill-X Multi-



Spill-X safety kits from EM Science.

purpose Treatment Kit has all one needs to contain spills involving acids, caustic solutions and solvents. The Spill-X Caustic and Spill-X Acid Treatment Kits can be used to neutralize and solidify caustic solutions and acid spills, including hydrofluoric acid. Agents in the Spill-X Solvent Treatment Kit are designed to both adsorb solvent spills and elevate the flash-point above 140 °F, says EM Science. Completing the line-up is a kit for formaldehyde spills. All kits are supplied with safety goggles, protective gloves and instructions — all contained within either

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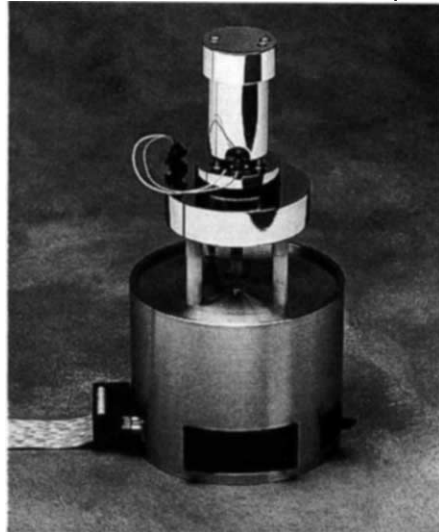
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a wall-mounted or portable case.

The Spill Containment Pack from Whatman contains all the reagents and materials necessary to contain leaks and spills (*Reader Service No. 113*). The £22.50 (UK) **spill containment kit** includes large booms to limit the spread of hazardous materials, compact booms and loose sorbent fibre to assist in mopping up, plastic disposal bags, a scoop and a pair of nitrile safety gloves. The sorbent materials, which are non-toxic and inert, are specially treated to absorb aqueous and solvent liquids: up to 3 litres can be absorbed per 250 g of sorbent material, says Whatman.

Measuring devices

The NanoScope **electrochemical scanning tunnelling microscope** (ECSTM) from Digital Instruments allows simultaneous STM imaging and voltammetry with atomic resolution (*Reader Service No. 114*). The ECSTM, which combines the power of STM with a bi-potentiostat-controlled electrochemical cell, is available as a \$16,000 (US) stand-alone unit or as an attachment that plugs into the NanoScope II STM controller. Redox reactions, plat-



STM meets electrochemistry.

ing, electrofinishing and other electrochemical processes can be monitored in real-time with atomic resolution, says Digital. STM imaging can be performed *in situ*, with the potentiostat active, or *ex situ*, with the potentiostat inactive. The ECSTM system consists of a special microscope base, which houses the bi-potentiostat, STM scan head, software, and electrochemistry cell.

Packard has introduced the Tri-Carb 1900TR **liquid scintillation counter** that is equipped with a built-in computer to speed sample processing (*Reader Service No. 115*). The counter features time-resolved LSC background reduction technology, which cuts background in half, doubles sensitivity, reduces cocktail con-

sumption and saves on counting time, says Packard. The cassette-loaded, bi-directional conveyor mechanism has a capacity of either 408 standard 20-ml vials or 720 4- or 7-ml vials. The counter also features a multitasking operating environment with CRT monitoring on a manoeuvrable support arm, as well as a full alphanumeric keyboard installed in a pull-out drawer. The system simultaneously analyses data, counts, reports, plots, changes samples and performs display functions. In addition, it can support multi-user sample processing for up to 30 users.

Back to basics

The Electronic Pipetting Centre from Jencons is a hand-operated **dispensing unit** that can be programmed to dispense



Jencon's multi-mode liquid dispenser.

liquids over the range of 1 µl to 5,000 µl (*Reader Service No. 116*). The microprocessor-controlled pipetting centre allows the user to programme run protocols using the following modes: titrate, pipette, dispense, dilute and multi-dispense. Up to ten run protocols can be stored in the instrument's memory, protected from erasure by use of a Lock Code.

In January, New Brunswick Scientific will be launching a new line of LoZone **ozone-friendly ultrafreezers** that are CFC-free and operate down to -80 °C (*Reader Service No. 117*). Two models are available: the £5,720 (UK) chest model with a 400-litre capacity and the £5,940 (UK) 350-litre upright model. In both LoZone freezer models, the refrigerant and the insulation/cladding are CFC-free. Design features include a local and remote alarm system with audible/visual signals and battery back-up, easy-to-clean polished stainless steel interiors, and internal lids and doors. □

These notes are compiled by Diane Gershon from information provided by the manufacturers. To obtain further details, use the reader service card bound inside the journal. Prices quoted are sometimes nominal, and apply only within the country indicated.